INFECTION AND RESISTANCE

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INFECTION AND RESISTANCE

AN EXPOSITION OF THE BIOLOGICAL PHENOMENA UNDERLYING THE OCCURRENCE OF INFECTION AND THE RECOVERY OF THE ANIMAL BODY FROM INFECTIOUS DISEASE, WITH A CONSIDERATION OF THE PRINCIPLES UNDERLYING SPECIFIC DIAGNOSIS AND THERAPEUTIC MEASURES.

BY

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TO THE MEMORY OF

A. Z.

THIS BOOK IS AFFECTIONATELY
DEDICATED BY HIS
SON

PREFACE TO THE THIRD EDITION

THE subject of immunity shares with other branches of biology the difficulties, as well as the attractiveness, of being constantly subject to alteration of point of view and conception. The fact that it deals with problems closely related to medicine, while at the same time its observed phenomena represent, in many cases, fundamental biological laws, attracts workers of many varieties of training, and the evidence by which it grows, emanates from many different sources. The clinician, the statistician, the epidemiological field worker contribute equally with the laboratory investigator, and the development of the past decade has been particularly characterized by the increasing application of the methods of chemistry and physics to its problems. While our subject is, as yet, far from what we may speak of as an exact science, it has already made considerable headway toward this possible goal by enlisting the interest of biologists, chemists and physicists; and no one who is following the subject can fail to appreciate the far-reaching effects upon it that may be looked for from work like that of Landsteiner on the chemical alteration of antigens, and of Loeb's important studies on proteins and colloidal behavior.

The worker in immunity who would seek for new methods of approach cannot afford any longer to neglect the guidance of the fundamental sciences. At no time have the experimental possibilities of this subject been greater or more attractive, but at no time, also, has there been such a rapid readjustment of point of view in many of its phases. Failure to appreciate this would make a treatise like this book thoroughly antiquated and worthless in a few years.

In rewriting the book we have considerably altered the arrangement of material into what appears to us a more logical sequence. We have omitted the final chapter on colloids by Prof. Young, included in former editions, largely because excellent books on colloidal chemistry, such as those of Williams and of Bancroft, are now available, and because the principles of this subject are, at the present time, more generally familiar to students of infectious disease than they were formerly. A considerable part of the material bearing on this subject has been incorporated in the text wherever applicable.

All new material accessible to us which we have considered well founded on experiment and observation has been included, and new opinions and observations, though often still in the balance, have

been critically discussed whenever their bearing has seemed to us of sufficient importance.

The chapters on anaphylaxis have been completely rewritten. When the first edition was published, this phase of the subject consisted very largely of numerous exact, but uncoördinated observations. The work which has been done since that time has revealed relationships and a basis for an orderly classification of phenomena; and although many important points are still matters of controversy, it has been possible to treat the subject of hypersusceptibility in a more orderly and logical manner than was formerly possible. In subjects in which so many uncertainties are involved as in that of hypersusceptibility, it has often been necessary to express personal opinions, but whenever this has been done, we have endeavored to thoroughly discuss opposing views and to furnish the reader with a sufficient amount of material to formulate an opinion of his own; or at least to realize that legitimate differences of conception existed.

The final chapters on practical therapeutic methods and the theories upon which they are based, have been enlarged and rewritten with a purpose of making them more definitely useful to those engaged in the clinical and laboratory study of infectious disease. While we hope that the book, as rewritten, will prove more useful than formerly to physicians, public health workers and laboratory investigators, we have adhered particularly to the original purpose, namely, the preparation of a critical treatise for the use of students of medicine and public health. We are more than ever convinced, from our experience in teaching such students, that immunology can be presented with sufficient clearness and simplicity to make it easily accessible to students at this stage of their careers, and that a thorough survey of the subject is almost indispensable to a proper subsequent approach of the problems of infectious disease.

No important statement has been made without as thorough a review of the literature as we have been able to give it within the allotted space.

Purely practical laboratory methods have been detailed in only a few instances where they seemed essential to the understanding of the phenomena described, and, in the therapeutic sections at the end, sufficient detail of procedure has been given to guide the experienced practitioner; but no attempt has been made to supply with this book, in any sense, a manual of experimentation.

HANS ZINSSER.

February, 1923.

PREFACE

Infectious disease, biologically considered, is the reaction which takes place between invading microörganisms and their products on the one hand, and the cells and fluids of the animal's body on the other. The disease is the product of two variable factors, each of them to a certain extent amenable to analysis, and it is self-evident that no true understanding of this branch of medicine is possible without a knowledge of the biological principles which laboratory study has revealed.

For the purpose of helping to render such knowledge easily accessible this book was written. While it is hoped that it may prove useful to the practitioner and laboratory worker, it is intended primarily for the undergraduate medical student. To many it will seem that the subject in general and our method of treatment especially are too technical and difficult for this purpose. Our own experience contradicts this. During the past three years the writer has had the opportunity to deliver lectures and to give laboratory courses on this subject to medical students of 2d, 3d, and 4th-year classes at the Stanford and Columbia Universities. It has been a pleasant experience to find the medical student eager for the opportunity to obtain this knowledge and, under the present increased requirements for preliminary training at our best schools, fully capable of assimilating it. It is not a good plan to attempt too extensively to simplify material that, in its close analysis, presents complex phenomena and intricate reasoning. For this reason no attempt has been made to write an A B C of immunity as a quick road to comprehension. No true insight into any branch of medicine or, for that matter, into any other science, can be attained without a certain amount of labor; however the concepts of this subject are, indeed, relatively simple after the first principles have been mastered, and the writer has attempted, therefore, at the risk of seeming pedantic in places, to treat the subject critically, separating strictly those data which may be accepted as fact from those in which legitimate differences of opinion prevail.

As far as was feasible every chapter has been written as a separate unit. This has necessitated occasional repetition, but, it is hoped, will add considerable to clearness of presentation in each individual subject. Theories have been discussed with as little prejudice as the possession of a personal opinion in many cases has permitted.

The chapter on Colloids was written especially for the book by Prof. Stewart W. Young, of Stanford University. Since so many analogies between serum reactions and those taking place between colloidal substances generally have been observed, it has seemed best to devote this chapter entirely to the elucidation of the principles governing colloidal reactions, so that its contents may be utilized as explanatory of the many allusions made to colloids in the rest of the text.

All available sources of information have been freely used. In the large majority of cases we have had access to the original papers and monographs. However, we acknowledge much aid from careful reading of the admirable summaries, written by acknowledged authorities, in the works edited by Kolle and Wassermann, and by Kraus and Levaditi. Similar acknowledgment is made to equally important sources in Weichhardt's Jahresbericht, the Bulletins of the Pasteur Institute, and in such text-books as those of Paul Theo. Müller, Emery, Adami, Gideon Wells, Marx, Dieudonné, and others. It is needless to acknowledge the use of such classics as that of Metchnikoff or of the many critical writings of Bordet and of Ehrlich—masters who have helped to shape the thoughts of all men working in this field.

The writer takes pleasure in acknowledging many helpful suggestions from his associates, Drs. Hopkins and Ottenberg, and much aid, in the verification of references, from Mr. Walter Bliss, Fellow in the Department of Bacteriology.

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CHAPTER I

INFECTION AND THE PROBLEM OF VIRULENCE

The early history of our knowledge of infectious disease is that of fermentation. It was a philosopher, Robert Boyle, writing in the 17th century, who prophesied that the problem of infectious disease would be solved by him who elucidated the nature of fermentation. His prediction was fulfilled 200 years later by the train of investigations begun by Cagniard-Latour and by Schwann, and carried to a brilliant culmination by Pasteur. It was the discovery of the living nature of ferments and the specific nature of the various micro-organisms which caused the several forms of fermentation, and especially of putrefaction, which made possible rational investigations in the field of infectious disease and led by analogy, first to logical speculation—then to actual experimental proof of the etiological relationship between the minute forms of life and the communicable diseases.

It is not much more than 50 years since Pollender described the anthrax bacillus in the blood and spleens of animals dead of this disease. In this short period the large number of maladies of animals and human beings caused by micro-organisms belonging both to the varieties spoken of as bacteria and to those classified as protozoa has necessitated the segregation of this branch of knowledge into a separate chapter.

The period of etiological investigation is now approaching its maturity. The causative agents of most of the more common infectious diseases have been discovered, and the biology of many of the pathogenic micro-organisms has been thoroughly studied both in their artificial cultures and in the infected animal body. In spite of a considerable accumulation of facts, however, the science of immunity, that is, the study of the defensive powers of the living animal body against infection, is still in its infancy, and the practical therapeutic successes based on this science are disappointingly out of proportion to the really large amount of detailed knowledge of cellular and serum reactions at our disposal.

The study of putrefaction and of fermentation—though furnishing the basic analogy from which the first impulse was obtained—

presented after all a problem infinitely more simple than that of the infection of living tissues with bacteria. For, given any organic material containing suitable nutritive constituents, with favorable environmental conditions of moisture and temperature, and spontaneously or experimentally inoculated with germs of a proper species, and the phenomena which ensued were merely those of bacterial growth, in which an active part was played by the bacteria only, the dead organic materials serving simply as a passive menstruum for these activities.

During the earlier days of the development of bacteriology, therefore, when the attention of investigators was concentrated primarily upon the discovery of the specific causal agents of various infectious diseases, it seemed that the simple bringing together of pathogenic germ and susceptible subject should suffice for the accomplishment of an infection. We have learned, however, that the process is much more involved, and that, fortunately for the survival of the higher animals and man, the conditions which determine infection are intimately dependent upon a variety of secondary modifying factors.

Throughout nature bacteria are abundant, and the environment of man and animals, the outer integuments of skin and hair, and the mucous membranes of the conjunctivæ, the intestinal and respiratory tracts, are constantly inhabited by a thriving bacterial flora. The distribution of certain species in definite localities is often sufficiently constant to be regarded as a normal condition. Thus the *Bacillus xerosis* is a characteristic inhabitant of the conjunctiva, certain cocci and spirilla are always present in the mouth and pharynx, as is Döderlein's bacillus in the vagina. The fact that bacilli of the colon group are invariably present in the bowels of animals and man from the first few days or hours after birth has even been interpreted by some investigators as a physiologically beneficial condition. In the course of ordinary existence, therefore, and much more so during the course of accidental exposure to individuals in whom infection is present, the bodies of the higher animals are in intimate contact, not only with ordinarily harmless bacteria (saprophytes), but also with many varieties of the micro-organisms spoken of as *"pathogenic"* or disease-producing. Perfectly normal individuals have, then, on occasion, been found to harbor diphtheria bacilli in nose and pharynx, meningococci have been found in similar localities, and tetanus bacilli, the bacillus of malignant edema, the Welch bacillus, and other distinctly pathogenic germs have been isolated from the intestinal contents of individuals who showed no evidence of disease. In fact, the problem of the so-called bacillus carriers—persons who, though themselves apparently well for the time being, harbor within their bodies and distribute to their environment bacteria capable of causing disease in others—is, as we shall

see, now recognized as one of the most important difficulties of sanitary prophylaxis. In the case of typhoid fever this is particularly true, for it is now well known that a perfectly healthy individual may harbor typhoid bacilli in the gall-bladder for years and constitute, through all this time, a constant focus of danger to the public health.

The accomplishment of an infection, then, is not determined merely by the fact that a micro-organism of a pathogenic species finds lodgment in or upon the body of a susceptible individual, but it is further necessary that the invading germ shall be capable of maintaining itself, multiplying and functionating within the new environment. An infection, then, or an infectious disease, is the product of the two factors, invading germ and invaded subject, each factor itself influenced by a number of secondary modifying circumstances, and both influenced materially by such fortuitous conditions as the number or dose of the infecting bacteria, their path of entrance into the body, and the environmental conditions under which the struggle is maintained.

We have in truth, then, a battle of two opposed forces, the result of which is infectious disease. And it is the systematic analysis of these forces in their variable conditions, and the laws which govern them, which constitutes the science of immunity. It is the initial skirmish between the two which determines whether or not a foothold shall be gained upon the body of the subject and an infection thus established, and it is the balance between them which decides the eventual outcome of recovery or death. And though it is unfortunately true that much of the knowledge gained by such studies has yielded few direct therapeutic results, the facts that have been revealed are fundamental to the pathology of infectious disease and as essential to the clinical understanding of these maladies as is the knowledge of the mechanism of the circulation, the chemistry of metabolism, or the structural changes of the tissues to the comprehension of other pathological conditions.

And from this point of view the study of infectious diseases can be made an eminently logical one, in that, knowing the criteria which govern the infection of a human being with a given germ, knowing the probable path of entrance, manner of distribution, and biological activities of the micro-organism, and the peculiarities of the mechanism of resistance set in motion in the body by this particular infection, definite clinical deductions can often be made.

Pathogenicity and Saprophytism.—One of the most fundamental facts, immediately apparent on considering the problems of infection, is the phenomenon that among the innumerable varieties of bacteria and protozoa present in nature there is a very limited group which is capable of becoming parasitic upon the body of higher animals, and among these a still smaller proportion which is capable of being "pathogenic" or causing disease.

We have used the terms pathogenic and non-pathogenic as practically synonymous respectively with "parasitic" and "saprophytic." But, as we shall see, although as a rule a micro-organism must be parasitic to possess pathogenic powers, some of the true saprophytes or so-called half-saprophytes may be pathogenic under certain conditions, and the terms do not cover each other absolutely.

It is reasonable to suppose that all micro-organisms were originally in the condition which we designate by the term *"saprophytic."* By this term we imply that these germs maintain themselves only upon dead organic matter and do not thrive in or upon the living animal tissues. The class of saprophytes is widely distributed and constitutes, of course, the most important group of bacteria in nature, since upon the activities of these germs depends the unlocking of nitrogen and carbon from the organic complexes in the dead bodies and waste products of animals and plants. Such bacteria if strictly saprophytic, that is, entirely unable to maintain themselves upon living tissues, have little importance as producers of disease, or, expressed in technical terms, have little "pathogenicity." Nevertheless, there are cases in which strict saprophytes may cause disease by lodging upon and growing in animal tissues which have been killed by other causes, so-called necrotic areas; and these, still being in relation with the body as a whole through the blood and lymph channels, furnish an area of saprophytic growth from which products of putrefaction or even bacterial poisons may be absorbed. While, as a rule, the disease following the invasion of necrotic tissue—such as gangrenous amputation stumps, old unhealed sinuses, diabetically gangrenous areas, etc., may be caused by a large variety of saprophytic bacteria, there are a few very important and specifically pathogenic bacteria which are, strictly speaking, saprophytes. Thus the form of meat poisoning caused by the *Bacillus botulinus* is due entirely to the poison formed by this bacillus outside of the body within the substance of the dead foodstuff, and disease ensues as the result of subsequent ingestion of this poison with the food. In the same way the tetanus bacillus and, less strictly speaking, the diphtheria bacillus, at least in its ordinary mode of attack, are rather closer to the class of saprophytes than to that of the parasites, since neither of these bacteria, under usual circumstances, invades the substance of the tissues beyond the point of initial lodgment, causing disease only by the production of specific poisons, a condition known as "toxemia" or intoxication. The tetanus bacillus, moreover, is not usually capable of maintaining itself and multiplying even at the point of initial lodgment unless the tissues have been injured by trauma or irritated by the presence of foreign bodies. Bacteria of such characteristics, therefore, though pathogenic—that is, incitant of disease—remain nevertheless essentially saprophytes living upon the dead animal tissues,

not invading the living cells or body fluids. It is true that investigations of Frosch [1] have shown that diphtheria bacilli may often be found in blood and organs of diphtheritic patients, and tetanus bacilli have occasionally been found in the spleen. However, such distribution is not necessary for the production of disease by these bacteria, and the essential point remains that they may cause violent, often fatal, disease without truly departing from their saprophytic mode of life upon dead tissues. Between the saprophytes and the true parasites or invaders of living tissue many transitions occur, and the condition of parasitism is probably a form of specific adaptation.

How such transition may be biologically developed is probably well illustrated by the investigations of Italian bacteriologists upon tetanus bacilli.[2] Tarozzi [3] inoculated guinea pigs and rabbits with tetanus spores subcutaneously and found that these spores were rapidly transported to the liver, spleen, and kidneys, where they could maintain a latent existence for as long as 51 days. If during this period trauma or any injury of the organs was practiced which led to the formation of necrotic tissue the spores would develop upon this basis and cause acute or chronic tetanus. Canfora,[4] continuing these studies, likewise found that tetanus spores inoculated under the skin are rapidly distributed throughout the circulation. If no trauma has taken place at the point of inoculation the locally lodged spores may be rapidly destroyed, probably by phagocytosis. In the circulation they appear to be less rapidly eliminated and may be present for from ten to thirteen days. If, during this period, there is produced a small wound, blood clot, or necrotic area in the body—this may serve as a focus for development and tetanus may ensue. After ten or more days the spores disappear from the blood, but may then take up a latent existence in some of the organs—as stated by Tarozzi. Apart from their importance as constituting a sort of transitional condition between pure saprophytism and parasitism, these investigations would seem to have much bearing upon the so-called cases of "cryptogenic tetanus."

Resistance of Tissue Cells to Invasion.—True infection, that is, the invasion of one species by individuals of another, and the ability of the latter to multiply and functionate within the cell complexes of the former, is a process quite out of keeping with the ordinary plans of nature, throughout which there seems to be a distinct opposition to the colonization and functionation of one living being within the living substance of another. Thus, as

[1] Frosch. *Zeitschr. f. Hyg.*, Vol. 13, 1893.

[2] Belfanti, quoted from Canfora, *Centralblt. f. Bact.*, I. Orig. Vol. 45, 1908.

[3] Tarozzi. *Centralblt. f. Bact.*, Orig. Vol. 38, 1905.

[4] Canfora. *Centralblt. f. Bact.*, Orig. Vol. 45, 1908.

Bail [5] has pointed out, a mass of frogs' eggs will remain entirely uninvaded while alive, though the water surrounding it may swarm with bacteria of many varieties, but when by some accident such a mass of eggs ceases to live, it immediately falls prey to bacterial infection. The same point is illustrated by the rapidity with which intestinal bacteria will spread throughout the body after death, when during life they have remained confined to the lumen of the intestine, or, at most, get into the portal circulation, to be destroyed in the liver.

We must also bear in mind that invasiveness on the part of micro-organisms may take the form merely of an ability to lodge in the intercellular spaces. Secondary cell destruction, then, may be first from the outside as foci are established, and invasion of the cellular protoplasm in such cases follows only after toxic and pressure effects may have brought about cell death.

By the living cell, therefore, opposition is usually offered to invasion by bacteria, a vital function which Bail has attempted to make clearer by formulating it as a law, referring to it as "Das Gesetz der Lebensundurchdringlichkeit." Upon which cell function this vital resistance to invasion depends is to a large extent a mystery. It would seem to rest in principle upon the fact that the invading cell meets the invaded one under conditions peculiarly adapted to the activities of the latter, and is overcome before conditions suitable for its own activities have been established. The conditions here are not unlike those observed in the case of digestive enzymes, a comparison which becomes more than an illustrative analogy when we consider that apart from the mere mechanical disturbance created by the presence of bacteria as foreign bodies the struggle between invader and tissue is largely one of enzyme against enzyme. Thus, for instance, the gastric juice does not act upon the mucous membrane of the stomach during life—but after death, at autopsy, partial digestion of this membrane by the pepsin is often seen.

The function upon which the resistance of the living cell depends will probably not be understood until we have a clearer conception of what constitutes cell death. Morphologically, until decomposition has set in, a recently killed cell looks exactly like a living one, and the difference which permits one cell to continue metabolism and cell division, and inhibits this in another, is, so far at least, too intricate to yield to analysis. Of some interest in this connection, though vague, is the observation made by us some years ago, namely, that the degree of hydrogen ion concentration which changes the normally negative charge carried by bacteria in neutral solutions, to a positive one, corresponds roughly to the degree of acidity at which multiplication ceases.

[5] Bail. "Das Problem der Bakt. Infection." Klinkhardt, Leipzig, 1911.

Much more definite, and of greater possible significance are the more recent observations of Osterhout,[6] who has found that during the process of cell death electrical conductivity undergoes a definite change by means of which the process may be followed with precision. By means of careful experiments with, among other things, Laminaria, placed in an NaCl solution of a conductivity equal to that of sea water, he found within less than six minutes, a fall of resistance to 94.6 per cent. of the resistance it had in sea water. Replacement of the tissue in sea water brought about a return to practically normal resistance. His researches indicate that in the course of injury and death, electrical resistance diminishes progressively and if the exposure is sufficiently prolonged, death together with extensive fall of resistance occurs. If exposure to the injury is arrested sufficiently early, complete recovery of normal cell life and normal resistance are reëstablished. And of remarkable interest in this connection is that between the two, a degree of exposure to injury may be practiced which permits a recovery which is incomplete. It is by researches of this kind that we may hope eventually, perhaps, to gain some knowledge of the vital cell resistance to injury and invasion to which Bail has merely given a name. And it would be of the greatest interest to utilize the methods of Osterhout for similar studies on the effect of bacterial poisons of various kinds upon the resistance of body cells.*

Whenever this vital resistance or opposition is overcome, and micro-organisms enter the tissues or cells, an abnormal process is taking place, and this process is, strictly defined, infection. Nevertheless, it is by no means necessary that such infection should always be accompanied by manifestations of disease. It is true that, in most cases, the natural resistance is such that a struggle ensues by which the invader is destroyed or thrown off, or in which the invaded subject is functionally injured or even killed, and the accompanying evidences of such a struggle constitute what we know as infectious disease. But there are special cases, cases of adaptation, biologically speaking, in which neither invader nor host is seriously harmed.[7] In the field of protozoölogy, especially, there are many examples of true parasites, that is, invaders truly maintaining their metabolism at the expense of the tissues and body substances of the host, which do not arouse reactions sufficiently vigorous to be termed "disease." Thus the *Trypanosoma Lewisi* may be found in the blood of rats [8] without noticeably affecting the health of the ani-

[6] Osterhout. *Jour. Biol. Chem.*, 23, 1915, 67; *Proceed. Amer. Philosophical Soc.*, 55, 1916, 533; *Jour. Gen. Physiol.*, 3, 1920, 15, and 3, 1920, 145.

[7] See also Bail, *loc. cit.*

[8] Doflein. "Die Protozoen als Krankheitserreger."

* In this discussion, however, we must not forget that there are a number of diseases caused by micro-organisms which characteristically invade the cells

mals, and other protozoa have similarly been found in organs and blood stream of a number of other apparently healthy animals. Although such conditions have been frequently spoken of as "infection without infectious disease," the distinction is probably one of degree only—there being some reaction on the part of the host even in the mildest cases, if only in the weakening by withdrawal of body substance, which distinguishes the infected from the uninfected animal. In other cases there may even be advantage to the host, following the infection, to the detriment of the invading micro-organism, a phenomenon most clearly illustrated by the invasion of the root hairs of leguminous plants by the Nitrogen-fixing "root-tubercle" bacilli, a condition in which, as Fischer says, the plant may be regarded as parasitic upon the bacteria.

In cases of so-called chronic septicemia in which bacteria may be again and again isolated by blood culture from the circulation it is more than likely that the organisms are constantly present, not because they multiply or maintain themselves within the circulation, but rather because they are being continuously discharged into the blood from an established focus in the tissues—as, for instance, on a heart valve. We have examined the serum of patients with subacute and chronic septicemia (endocarditis), and often found powerful opsonic action against the invading germs even when the patient's own serum and leukocytes were used in the tests, evidence that the bacteria were probably being successfully disposed of after they had gained entrance into the blood stream. In rabbits, too, in our experience and in that of Miss Gilbert in this laboratory, it would seem that protracted septicemia is present only when secondary foci have been established from which the bacteria are constantly being discharged into the blood. This we believe is rather the rule and the establishment of a balance within the blood stream an exception. When bacteria *do* succeed in withstanding successfully the opposing forces active within the circulating blood their rapid accumulation, the collapse of the defensive mechanism, and death of the patient are probably the most common course.

The point of view which we have expressed in the preceding paragraph has been impressed upon us with particular insistence by the observation of certain cases of bacteriemia following infections of the middle ear, mastoid processes, and thromboses of adjacent veins. In such cases it appears that the blood may be flooded with bacteria which, nevertheless, disappear after the focus of infection has been

of the body directly. Such is the case of the malarial plasmodia, with some of the Leischmannia, which cause Kala-Azar, which enter the endothelial cells of the spleen, liver and bone marrow, and is also characteristic of the Rickettsia bodies associated with typhus and trench fever. This also probably applies to some of the filtrable viruses, notably those of smallpox, encephalitis and herpes.

removed. We have recently had the opportunity to observe this in a case of septicemia caused by *Streptococcus mucosus,* in which blood culture plates showed very numerous colonies, and in which recovery followed promptly upon complete excision of the thrombosed veins. It would seem to us, therefore, that bacteriemia offers a rather better prognosis than was formerly supposed, at least in cases in which the focus is surgically accessible.

The same principle is illustrated in the ordinary clinical course of typhoid fever in the human being. Here the disease begins as a bacteriemia. Very rapidly, usually within two weeks, the bacteria disappear from the blood stream and a high serum immunity is established in the patient. Nevertheless, the bacteria remain actively growing within definite foci in the tissues, where they are to a certain extent protected or inaccessible to the defensive powers so successfully active in the blood stream. At any rate the patient remains diseased and the bacteria can be isolated from the spleen, gall-bladder, and intestines at a stage when they are no longer present in the blood stream, and during which a measurement of the bactericidal and opsonic powers of the patient will reveal a serum immunity much higher than normal. Just why the organisms are protected from these influences in certain tissues and not in others we do not know.

The Problem of Adaptation in Infection.—We have seen, then, that a micro-organism may be pathogenic and still be purely saprophytic in its mode of life. In order that this can occur, however, it is necessary that it should possess the power of producing at the place of lodgment a poison or toxin which can be absorbed and cause disease. The condition which ensues is not, properly speaking, an infection, but rather a *"toxemia,"* differing from the toxemias resulting from the ingestion of drugs or other poisons only in so far as the toxins are manufactured at some point of bacterial lodgment within the body of the victim. Typical tetanus and diphtheria, for instance, can be produced as readily by injection of the bacteria-free culture filtrates as by inoculation with the bacteria themselves. And although these bacteria may, on occasion, become invasive and thereby satisfy the criteria of true infection, this is not necessary for their pathogenicity.

In the large majority of bacterial diseases, however, it is necessary that the germs shall be capable of producing a true infection before they can become pathogenic, and it is our task therefore to attempt to analyze those bacterial attributes upon which the invasive power or virulence may be said to depend.

In the realm of infectious micro-organisms a wide range of cultural variations is encountered which indicates that some of these germs have adapted themselves very closely to the specific environmental conditions found in the living animal body, while others can

take up with ease and under the simplest cultural conditions a purely saprophytic existence.

Many pathogenic micro-organisms have so far defied all attempts at cultivation in artificial media. These we cannot use for examples since it may well be that the failure of attempts in many of them may hinge upon such simple alterations of method as the exclusion of oxygen, the addition of fresh tissue, or the supplying of amino-acids, which have made possible the cultivation of the spirochæta pallida and the leprosy bacillus. But among those which we can cultivate there are many which require for successful cultivation the production of artificial conditions simulating closely those obtaining in the living body. Thus malarial plasmodia can be made to multiply only if furnished with uninjured human red blood cells, within which they can develop. The gonococcus requires, in its first cultures outside the body, a medium containing human protein; and the hemophile bacteria, among them the influenza bacillus, require hemoglobin. Other organisms like pneumococci, many streptococci, diphtheria bacilli, and many others, though easily grown on artificial media, are still fastidious in their requirements and develop sparsely or not at all unless definite conditions of nutrient materials, temperature, reaction, and osmotic pressure are observed. On the other hand, typhoid, anthrax, and dysentery bacilli, staphylococci and numerous other pathogenic germs grow easily and luxuriantly on the simplest laboratory media and within a wide range of environmental variations.

Biologically considered, we could arrange the scale of adaptation to parasitic conditions on this basis and it would seem, a priori, that those bacteria which had thus adapted themselves most closely to the living body should be the most infectious. There is not, however, such parallelism, since many of the most powerfully invasive or virulent germs, for instance, the anthrax bacillus, have retained their capacity for saprophytic life to the fullest extent. It is more logical, therefore, to classify parasites, not according to their ability to revert to saprophytic conditions, but rather, as Bail [10] has done it, on the basis of their relative powers of invading the living body. His classification, of course, implies that the position of each micro-organism in this scale must be determined with reference to a given animal species, since a germ which is highly infectious ("parasitic" in Bail's sense) for one species may be a "half-parasite" or even a pure saprophyte for another.

Briefly reviewed, his classification is as follows:

I. *Pure Saprophytes.*—(Necroparasites, superficial parasites, or external parasites.)

Micro-organisms which under no circumstances can be made to develop within the living tissues of a given animal. This does not

[10] Bail. *Loc. cit.*

exclude their pathogenicity for this animal, since, like the diphtheria or tetanus bacillus, they may develop and produce toxins on the basis of a localized area of dead tissues.

II. *Pure Parasites.*—Organisms like the anthrax bacillus or the bacilli of the hemorrhagic septicemia group which, implanted in small quantity in an animal, will rapidly gain a foothold, thrive, and spread throughout the body.

III. *Half parasites,* organisms which may be infectious if introduced into the animal body, but, not possessing this invasive power to the same degree as the preceding class, require the inoculation of considerable quantities, often a special mode or path of inoculation, or even possibly a preliminary reduction of the local and general resistance of the infected individual in order that they may multiply and become generalized. This class includes the large majority of the bacteria pathogenic for man.

This property of invasive power is spoken of as *virulence* in contradistinction to *toxicity*—the latter implying merely the ability to produce poisons, and not necessarily being associated with the power to invade.

However we may classify these conditions (and it must be remembered that a classification of complex scientific facts should never be regarded as a rigid definition of limitations, but merely as a convenient working basis), the facts of the matter are that many infectious micro-organisms, like, let us say, the treponema pallidum and the gonococcus, have adapted themselves so rigidly to the human body in cultural and other requirements, that they cannot be cultivated outside the body with any degree of success, and can be so cultivated only when, in nutritive and temperature conditions, the circumstances prevailing in the human body are to some extent simulated in the cultural procedure. This is particularly true of many of the filtrable virus varieties and the protozoan infecting agent. When such organisms can be cultivated, as, for instance, the gonococcus and the influenza bacillus, prolonged cultivation upon artificial media gradually increases their ability to grow upon them. Conversely, with this increased ability to grow outside the body more easily and in simpler media, as a result of prolonged cultivation, the pathogenicity may often decrease.

While both of these facts are true of individual cases, still they do not constitute a general rule. Many organisms, like the anthrax bacillus, typhoid bacilli, plague bacilli, cholera spirilla, and a host of others, can invade the bodies of one or more species of animals with great violence and still retain the capacity for a saprophytic existence to the fullest extent, are easily cultivated on artificial media and unless specific efforts are made to reduce their virulence, they retain their infectious power almost indefinitely if preserved on artificial media. Thus, virulent cultures of anthrax, of plague,

of typhoid bacilli, etc., can be kept in sealed tubes, in the cold and in the dark, almost indefinitely, without losing either their ability to grow or their ability to cause specifically characteristic infection when again introduced into the susceptible animal. The evidences of special adaptation of different micro-organisms to particular species of animals is dealt with extensively in the chapter on Natural Resistance.

Of the greatest importance in illustrating the adaptation factor in infection is the ease with which *fluctuations of virulence can be artificially produced* in certain species of bacteria. The ease with which such variations can be produced would seem to furnish another argument in favor of the conception that the property of invasiveness is a biological attribute of relatively recent acquisition. Such variations are particularly noticeable in the cases of streptococcus and pneumococcus, organisms in which no two strains need be alike in infectiousness, and in which the injection of some strains into susceptible animals may produce no result whatever, while other strains will kill if administered in the smallest measurable quantities. As a rule, the virulence of such strains can be enhanced for a given species of animal by successive passages through animals of this species. In the case of streptococci, strains which may not kill mice in quantities of a cubic centimeter or more, of an 18-hour broth culture, can, by many passages from mouse to mouse, be so enhanced that one-one hundred thousandth of a cubic centimeter of a similar culture will kill promptly. This cannot be done with all strains of streptococci, and of a dozen strains or so, one may succeed with two or three only.

Among the earliest observations on this point are those of Pasteur [11] in his work on rabies. He found that the virus of hydrophobia when successively passed through rabbits gained in virulence until a degree of maximum infectiousness was attained beyond which it could no longer be enhanced. After only three passages through monkeys, however, the virulence of this "virus fixe" for rabbits was reduced almost to extinction. His experience with swine plague was similar. Swine plague bacilli successively passed through rabbits and pigeons gained enormously in virulence for these animals respectively, but lost in virulence for hogs.

There are numerous methods, moreover, by which the virulence of micro-organisms can be attenuated by laboratory manipulations, and since many of them are of great importance in the active immunization of animals we will reserve their detailed discussion until we come to consider the methods of immunization themselves. Suffice it to say in this place that most methods of attenuation consist in subjecting the bacteria, in artificial culture, to deleterious influences, either of unfavorably high temperature, exposure to light

[11] Pasteur and Thuillier. *Compt. rend. de l'acad. des. sc.*, Vol. XII, 1883.

or harmful chemical agents, or allowing them to remain in prolonged contact with the products of their own metabolism by infrequent transplantation. As a rule the attenuation which inevitably follows any form of artificial cultivation in the case of bacteria like streptococci or pneumococci can be delayed by preserving them in media containing sera or tissues. In the case of the pneumococcus, for instance, one of the best methods of conserving virulence in storage is to keep them either in a soft rabbit-serum-agar mixture, as practiced by Wadsworth, or, better still, to store them within the spleen of a mouse dead of pneumococcus infection, as recommended by Neufeld. The mouse is autopsied and the spleen kept in the dark and cold in a desiccator, under sterile precautions. This, again, as well as the enhancement of virulence on passage through the same species of animal—or the reduction of virulence for one species by passage through another—shows that such fluctuations are dependent upon a very delicate biological adaptation.

It is interesting, moreover, to look upon this process of adaptation as a sort of immunization of the bacteria against the defensive powers of the host, a conception early suggested by Welch. For just as the animal body may become more resistant to the offensive weapons of the invaders, so it is reasonable to suppose that the bacterial body may gradually develop increased resistance to the defensive mechanism of the host. And this, if it occurs, would of course lead to an increase of its invasive power or virulence. The increase of virulence by passage through animals would alone lead us to suspect that such acquired resistance to destructive agents on the part of the bacteria might be responsible for the enhancement, but additional evidence pointing in this direction has been brought by experiments in which it was shown that bacteria cultivated in the serum of immune animals not only gained in resistance to destruction by the serum constituents, but at the same time were rendered more highly pathogenic. Experiments of this kind were carried out by Sawtchenko,[12] by Danysz,[13] and by Walker.[14] The results of Walker are especially instructive. He worked with a typhoid bacillus which he cultivated for a number of generations upon the serum of a typhoid-immune animal, and found that after such treatment the organism had gained in virulence and lost in agglutinability by immune serum, and that a larger amount of specific immune serum was necessary to protect animals against it than sufficed for protection against normal typhoid strains not thus cultivated. We will refer to these results in a later chapter, since the conception will be easier to grasp when we have considered more

12 Sawtchenko. *Ann. Past.*, Vol. 11, 1897.
13 Danysz. *Ann. Past.*, 14, 1900.
14 Walker. *Jour. of Path. and Bact.*, Vol. 8, 1903.

fully the mechanism of defence by which the animal body is protected against invasion.

That this power of gaining resistance against deleterious influences on the part of bacteria is not confined to their resistance to the animal defences alone is well shown by the experiments of Danysz [15] upon the immunization of anthrax bacilli against arsenic. In inoculating series of 50 tubes containing arsenic dilutions (ranging from 1 to 10,000 to 1 to 200) with anthrax bacilli Danysz found that up to 1 to 5,000 the arsenic increased the growth of the bacilli; in concentrations higher than this growth was inhibited. By gradually progressive cultivation of the organisms in increasing concentrations of arsenic he finally succeeded in obtaining growth in solutions five times more concentrated than those in which they would develop at first.

It is intensely interesting also that Danysz found, both in the case of his serum-resistant and arsenic-resistant strains, that, as they became less sensitive to the deleterious effects of these agencies, they were altered morphologically in that they developed capsules. Similar in significance to this is the very important observation that certain strains of spirochæta pallida may acquire resistance against salvarsan or "606." [16] These so-called arsenic-fast strains are apparently unaffected by the injection of this preparation into the patient.

Relationship of Infectiousness to Capsule Formation.—The experiments of Danysz were probably the first to call attention to the possible relationship of bacterial capsule formation to virulence, and this particular phase of the subject has since then been extensively studied. It is a matter of common observation that microorganisms like the pneumococcus, the anthrax bacillus, some streptococci, and a number of other germs which are capable of producing capsules under suitable conditions are most virulent in the capsulated stage. As the strains are passed through animals and their virulence increases their ability to form capsules becomes more and more apparent—whereas the diminution of virulence which takes place on artificial media is accompanied by a gradual loss of capsule formation. Organisms like the Friedlander bacillus which retain their ability to form capsules almost indefinitely in artificial culture moreover do not lose their virulence to any great extent as long as this property is preserved. It is also well known that capsulated bacteria are peculiarly insusceptible to the ordinary agglutinating powers of specific immune sera. This has been noticed, not only in the case of heavily capsulated bacteria like those of the Friedlander group or the streptococcus mucosus, but, in the case of plague bacilli—where capsulation is usually present only in cultures taken

[15] Danysz. *Loc. cit.*
[16] Oppenheim. *Wien. kl. Woch.*, 23, 1910, No. 37.

directly from the animal body and cultivated at 37° C., Shibayama [17] has found a direct relation between non-agglutinability and a slimy condition of the cultures. Cultures kept at 5° to 8° C. in the ice-chest were easily agglutinable and lacked the slimy property. Cultures kept at 37.5° C. were slimy and thready in consistency and were not as easily agglutinated by the same immune serum. Porges [18] later showed that inagglutinable, capsulated bacteria can be made amenable to the agglutinating action of the serum—which we may assume to indicate vulnerability by the serum if the capsule is previously destroyed by heating at 80° C. for about 15 minutes in ¼ normal acid.

Against the cellular defences, the leukocytes, capsulated bacteria seem also to be more resistant than are the non-capsulated. This has been especially studied by Gruber and Futaki,[19] who find that a capsulated bacillus is rarely taken up by a phagocyte even when these cells are apparently normal and able to take up the uncapsulated organisms. They go so far as to claim that, in the case of anthrax in rabbits, the development or absence of a capsule determines whether or not infection can take place. The same conclusion is reached in similar studies by Preisz,[20] who does not believe that anthrax bacilli can ever cause infection unless they possess the power of forming capsules. All this experimental evidence points strongly toward a probable direct relationship between capsule formation and virulence, in the sense that a thickening of the ectoplasm may in some way protect the bacteria from the destructive forces aimed at them by the cells and fluids of the invaded body.

As a matter of fact, even when no distinct capsule is visible, it is nevertheless possible that ectoplasmic changes may take place. This phase of the subject has been thoroughly discussed by a number of writers, more especially by Eisenberg.[21] It appears that many bacteria, in which true capsule formation has not been observed, may show swelling or enlargement under conditions in which their offensive activities in the infected animal body are called into play.[22] Radziewsky [23] has noticed such swelling of B. coli in fatal guinea-pig infections, and spoken of it as "one of the characteristic signs of infectiousness." Kisskalt [24] has described the same thing in the case of streptococci, and Eisenberg interprets this as signifying an ectoplasmic hypertrophy comparable in principle to capsule formation. He looks upon the ectoplasmic zone as a protective layer, and

[17] Shibayama. *Centralbl. f. Bact.*, Orig. Vols. 38, 1905, and 42, 1906.
[18] Porges. *Wien. klin. Woch.*, p. 691, 1905.
[19] Gruber and Futaki. *Münch. med. Woch.*, 6, 1906.
[20] Preisz. *Centralbl. f. Bakt.*, Vol. 49, 1909.
[21] Eisenberg. *Centralbl. f. Bakt.*, I, 45, 1908, p. 638.
[22] These forms Bail has spoken of as "thierische Bazillen."
[23] Radziewsky. *Zeitschr. f. Hyg.*, Vol. 34.
[24] Kisskalt. Cited after Eisenberg, *loc. cit.*

calls attention to the observation of Liesenberg and Zopf,[25] who showed that capsulated strains of leukonostoc mesenteroides will withstand 85° C., a temperature at which uncapsulated forms are rapidly killed.

Upon this point our own recent work has seemed to us of considerable interest, although we cannot yet prove our point. In another part of this book (see page 110), we have described substances that are probably not whole proteins, which are rapidly exuded from all bacteria that we have examined, into the surrounding fluid. These so-called residue antigens, in spite of their apparent low molecular structure, react powerfully with antibodies and represent, we believe, metabolic products which ooze out from the bacteria as the result of their own chemical exchange. From bacteria that are capsulated, like the pneumococcus, from meningococci which are difficult to subject to agglutination, these substances can be obtained by simply washing the bacteria. In the case of other bacteria, like typhoid bacilli, tubercle bacilli, etc., it is necessary to grind and extract the bacteria in order to obtain them. It is our opinion that in capsulated bacteria these substances form a protective layer about the bacterial cell which, because of its ability to unite with antibodies, can divert these sensitizing substances from the bacterial cell itself.

Acquired Resistance to Antibody Effects.—Pertinent, also, to this discussion of adaptation is work in which the actual resistance of micro-organisms to reaction with antibodies has been shown to result from prolonged contact of the bacteria with blood or exudate from animals or human beings or with immune sera.

This seems to be shown especially by the experiments of Walker and Morishima, [25a] which are referred to in other places, in which it was found that bacteria grown on immune sera gain a certain amount of resistance against the injurious properties of these substances, and evidence more directly bearing upon the question is furnished by the studies on the typhoid carrier state in rabbits made by Chirolanza,[26] Blackstein,[27] Johnston,[28] and recently by Gay and Claypole.[29] The last-named writers found that they could regularly produce the typhoid carrier state in these animals if they first cultivated the typhoid bacilli upon a medium containing defibrinated rabbits' blood. Even in these cases, however, it is not at all improbable that the typhoid bacilli establish a permanent focus from which they are discharged into the blood stream.

[25] Liesenberg and Zopf. *Centralbl. f. Bakt.,* Vol. XII, 1892.
[25a] Morishima. *Jour. of Bacter.,* 6, 1921, 275.
[26] Chirolanza. *Ztschr. f. Hyg.,* Vol. 62, 1909.
[27] Blackstein. *Bull. Johns Hop. Hosp.,* 1891.
[28] Johnston. *Journ. Med. Res.,* 27, 1912.
[29] Gay and Claypole. Arch. of Int. Med., 12, 1913.

It is not likely, however, that this merely passive increase of the resistance to injury on the part of the bacteria accounts for the entire train of phenomena included in an enhancement of virulence. It has been suggested by a number of observers that definite active offensive characteristics distinguish the virulent from the avirulent bacteria, in that the former may secrete, within the living body, substances by which the destructive powers of serum and leukocytes are neutralized or held at bay. A very definite suggestion of such a possibility we find expressed in the now classical paper of Salmon and Smith [30] on hog cholera immunity, published in 1886. They say: ". . . the germs of such maladies are only able to multiply in the body of the individual attacked, because of a poisonous principle or substance which is produced during the multiplication of these germs." [31] Bouchard formulated such a theory in 1893 by speaking of the "produits sécretés par les microbes pathogéniques," substances which he found in cultures of virulent bacteria, and which seemed to reënforce the invasive powers of the germs. Kruse [32] also within the same year developed a similar idea. He assumed that bacteria may secrete enzyme-like substances which paralyze the destructive properties of animal serum, and in this way gain the power to invade. As a matter of fact we have learned, since that time, that staphylococci may secrete soluble substances, "leukocidins," which injure white blood cells, and that many bacteria produce similar poisons, "hæmotoxins," which specifically injure red blood cells—thereby causing anæmia and reducing the resistance of the host. However, the correlation and further elaboration of these thoughts of Salmon and Smith, of Bouchard and of Kruse was left to Bail,[33] in what is known as his "aggressin theory." Bail maintains on the basis of careful experimentation that virulent bacteria can produce within the animal body substances which he calls "aggressins," upon which depend their invasive powers or virulence. These substances are secreted only under stress of the struggle against the unusual defences, are not demonstrable in test-tube cultures, and are in themselves, according to Bail, entirely non-toxic.

He obtains these aggressins by injecting virulent bacteria into the peritoneal cavity of a guinea pig and immediately after death removing the exudate. This he centrifugalizes, removes the bacteria and cells, and sterilizes the supernatant liquid by the addition of small quantities of chloroform. The action of the exudates in which aggressins have been produced by the bacteria is the fol-

[30] Salmon and Smith. *Proc. Biol. Soc.*, Washington, D. C., III, 1884, 6, p. 29.

[31] A typewritten copy of this paper was kindly put at my disposal by Prof. Theobald Smith.

[32] Kruse. *Ziegler's Beiträge*, Vol. XII, 1893.

[33] Bail. *Archiv f. Hyg.*, Vols. 52 and 53, 1905; *Folio serologica*, Vol. 7, 1911.

lowing: (We take this tabulation from Bail's own paper on typhoid and cholera aggressins in the *Archiv für Hygiene,* Vol. 52, p. 342.)

1. Sublethal doses of typhoid bacilli or cholera spirilla become lethal when the aggressin is injected with them.

2. Lethal doses of bacilli which ordinarily would cause a slow infection only cause a rapid and severe infection when aggressins are added.

3. The addition of aggressin neutralizes the bacteria-destroying power of immune serum in the peritoneal cavity of a guinea pig.

4. The injection of aggressin alone produces subsequent immunity.

It is impossible to discuss with completeness the arguments advanced for and against the correctness of Bail's views until we have described in detail the mechanism of protection at the disposal of animals. But the main objection brought against this theory is that of Wassermann and Citron,[34] who claim that all these properties of the aggressive exudates can be explained by the fact that they contain extracts of the bacteria (endotoxins), which, injected with a sublethal dose of bacteria, merely enhance their action in the same way that this would have been accomplished by the injection of additional dead bacterial bodies. It will require much further work before this point is settled, and the problem is peculiarly involved and difficult. However, the recent work of Rosenow [35] on pneumonococci seems to bring some reënforcement to the ranks of those who maintain the existence of a special offensive substance at the command of virulent bacteria. Rosenow extracted pneumococci grown on serum broth and found that such extracts when made from virulent strains would protect avirulent strains from engulfment by phagocytes. The non-virulent strains left in these extracts for 24 hours became virulent. He believes, therefore, that the virulence of pneumococci depends largely upon the possession of these substances which he calls "virulins," and which in function at least are conceived as very similar to the "aggressins."

Recent results obtained by the writer [36] with Dwyer seem to indicate that anaphylatoxins produced from the typhoid bacillus possess some of the properties claimed for his aggressin by Bail. It is not impossible that the "aggressins" obtained by him were of this nature.

Virulence, then, may be analyzed into two main attributes: one a purely passive property of resistance or self-preservation on the part of the bacteria, perhaps morphologically expressed in ectoplasmic hypertrophy and capsule formation; the other an actively offensive weapon in the form of substances of the nature of the "ag-

[34] Wassermann and Citron. *Deutsche med. Woch.,* Vol. 31, 28, 1905.
[35] Rosenow. *Jour. of Inf. Dis.,* Vol. 4, 1907.
[36] Zinsser and Dwyer. *Proc. Soc. Exp. Biol. and Med.,* Feb., 1914.

gressins" of Bail or the "virulins" of Rosenow. The extent of our present knowledge of details does not warrant a statement of the case in more definite terms.

From what has been said above, then, it appears that mutual adaptation in the biological sense between the invading germs and the invaded body must play an extremely important rôle, not only in determining whether or not an infection is to take place, but also in influencing the degree of infection, its acuteness or chronicity, and through these facts, the eventual outcome. That many different types of adaptation may occur and that the balance struck must depend to some extent upon the relative speed with which either the invaders or the invaded can modify their biological properties to the new conditions, is obvious. Theobald Smith[37] has given this problem the most thorough analysis. A micro-organism that has been closely adapted to the human body may have developed particularly its offensive and defensive powers to such a degree that when it enters a new human body, it proceeds on its invasive course with speed and violence, and the infected tissues react with a powerful effort, to rid themselves of the foreign invader. The result is violent and acute inflammation and disease. This violent reaction on the part of the body would be evidence of a mobilization of protective functions to a living foreign substance to which no adaptation has taken place, and which the body attempts to get rid of. As long as this reaction on the part of the bacteria carries the upper hand, the increase in virulence will continue. Conversely, however, as Theobald Smith points out, the chronicity of a disease is largely dependent upon an adaptation between host and invader in which the reaction to the parasite is less violent, and in which a condition approaching more and more closely to a sort of symbiosis, is established. As Smith points out in his recent publication, "there is a struggle on the part of the parasites to adapt themselves and to establish some equilibrium between themselves and their host"; and, again, "the final outcome is a harmless parasitism or some disease of little or no fatality, unless other parasites complicate the invasion."

Such an adaptation probably takes place. On the other hand, it is not likely to occur unless from the beginning the balance is such that host and invader may be in contact for a period longer than duration of the ordinary acute infectious disease. In cases where the organism is either rapidly gotten rid of, or in which acute death ensues within a short time, such developments can hardly be expected. However, through gradual transitional stages and many successive infections, the condition might be produced from an originally acute form of disease to a more subacute or chronic one.

[37] Smith, Theobald. *Trans. Soc. Amer. Phys.*, 1921, see also *Jour. A.M.A.*, May, 1913, 60.

Such a conception would assign the slow and gradual but progressively invasive powers of such diseases as tuberculosis, leprosy, and syphilis in which systemic symptoms are manifest only after the disease has gained an extensive foothold, to the lack of acute physiological reaction resulting from the presence of the invading micro-organism. In the case of such infections as those caused by some of the yeasts or blastomyces we have seen foci of blastomycotic lodgment in the kidney and other organs surrounding which there was neither an accumulation of mobile cells—(leukocytes or lymphocytes)—nor any evidence of cloudy swelling or other injury, by poisons, of adjacent parenchyma cells. Here, as in tuberculosis or leprosy, the reaction induced by the presence of the micro-organisms is slow and gradual—expressed in an eventual fixed tissue-cell reaction and giant-cell formation—similar to that induced by insoluble foreign bodies. And it may well be that the progressive ability to multiply without arousing the invaded body to rapid and powerful reaction may account for the prolonged period of apparent well-being in the early stages of such infections and permit the invaders to pervade the body so extensively.

Criteria which determine Actual Infection.—In order that a micro-organism may be a true parasite in Bail's sense—or invasive—for any given species of animal it must of course possess certain basic cultural attributes which enable it to grow in the environment furnished by the host. For instance, a micro-organism which does not grow at temperatures below 37.5° C. cannot very well become parasitic upon cold-blooded animals. An excellent illustration of this influence of body temperature upon the invasive powers of bacteria is furnished by the different races of acid-fast bacilli which invade the bodies of man and of birds. The avian tubercle bacillus, for instance, is non-pathogenic for man and in cultures will not develop at temperatures below 40° C., which is about the body temperature of most birds. The human tubercle bacillus, on the other hand, is non-pathogenic for birds and ceases to grow in artificial cultures when the temperature is raised above 40° to 41° C. This is merely one of a number of examples which might be cited to demonstrate the necessity of simple cultural adaptation, as it influences the property of virulence. Again, it is probable that in order to develop in the animal body it is necessary that a micro-organism shall be capable of developing with little or no free oxygen. While this point is not definitely certain, it is not probable that any of the virulent bacteria can be strict aerobes. As a matter of experience none of the pathogenic bacteria at present known are absolute aerobes—though many of them grow better in artificial culture when oxygen is freely present than when it is absent.

The invading bacteria, then, must be culturally and in other respects adapted to development in or upon the tissues of the invaded

subject—a general capacity which is prerequisite to the property of *"pathogenicity."*

Furthermore, the conditions encountered by bacteria as they enter the animal body will vary considerably according to the *path by which they gain entrance.* Organisms entering by the intestinal canal are subjected to conditions of acidity or alkalinity, the action of digestive juices, of bile, and to competition with other intestinal bacteria, forces to which many pathogenic germs will succumb, while others may survive there and thrive. Those entering into the tissues by way of the skin and mucous membrane, on the other hand, encounter an immediately mobilized protective mechanism which, successfully resisted by some of them, might easily and quickly dispose of small quantities of other bacteria more resistant to conditions in the bowel. It is but natural for this reason that the accomplishment of an infection by any given germ must depend to a great extent upon its gaining entrance to the body by the path best adapted to its peculiar requirements.

The mechanical protection afforded by the coverings of skin and mucous membranes is as a rule sufficient to prevent the penetration of any bacteria which by chance may have found lodgment upon them. In the case of the most usual pyogenic cocci and many bacilli such protection is probably absolute, and a distinct break of continuity, such as a bruise or a wound, even though this may be too small to attract attention, is necessary for successful infection. In the case of a very limited number of diseases infection seems to take place even through the unbroken skin, and the method, often spoken of as the vaccination method of Kolle, employed in many instances when it is desired to produce experimental plague infection in rats or guinea pigs, consists in merely rubbing a small amount of cultural material into a shaven area of the skin. However, in this case, as well as in other instances where mere massage of bacteria into unbroken skin has led to successful inoculation, it is more than likely that success has depended upon either microscopic lesions or possibly the violent introduction of the organisms into the sebaceous glands, the sweat glands, or hair follicles. The defence of intact mucous membranes, however, is by no means impervious. While many organisms can be implanted upon mucous membranes with impunity, there are a number of others that can cause local inflammations upon these and can further pass through them into the deeper tissues and thence into the general system. Thus gonorrhea is ordinarily a disease of implantation upon a mucous membrane, and diphtheria bacilli and streptococci give rise to localized disease on the pharyngeal and nasal mucosæ, the latter not infrequently penetrating from the initial point of lodgment upon the mucosa into the deeper tissues and the circulation, causing a condition of "septicemia" or "bacteriemia." For the experimental determination of

the penetrative power of organisms through mucous membranes the conjunctiva has been a favorite test object, and it has been shown that plague [38] and glanders,[39] as well as hydrophobia, may be transmitted by simple instillation of infectious material into the uninjured conjunctival sac. In the case of hydrophobia [40] it is related that in Paris a young man contracted hydrophobia by rubbing his eyes with a finger contaminated with the saliva of a rabid dog. In the case of syphilis, though often claimed, there is no positive proof to show that infection may take place through the uninjured surfaces. It has been definitely shown, however, that tubercle bacilli [41] may pass into the lymphatics through the intestinal mucosa without there being any traceable injuries on this membrane.

It may well be, however, that even without the existence of demonstrable morphological lesions penetrability by micro-organisms may presuppose local physiological or functional injury, such as congestion or catarrhal inflammation.

Thus it is seen that the mechanical obstacle to the entrance of micro-organisms offered by skin and mucous membranes, though important and not to be underestimated, is by no means a perfect safeguard.

However, it is only very definite species of micro-organisms which can cause disease at all when introduced into the body by these paths. For, although the rubbing of plague bacilli into the skin, or the inoculation of a cut surface with streptococcal or glanders bacilli, will rapidly lead to progressive infection, similar inoculation with the typhoid bacillus or the cholera spirillum would lead to no such result. And, though the swallowing of pus cocci, pneumococci, and a number of other micro-organisms would be entirely without effect, similar ingestion of the typhoid and cholera organism would usually result in typical infection.

The path of introduction, therefore, is an important consideration in determining whether or not a given micro-organism may give rise to disease. It is necessary that the manner of gaining entrance be suited to the cultural and other peculiarities of the germ in question. In the case of cholera, for instance, the spirillum which causes this disease is peculiarly susceptible to the deeper defences residing in the body fluids and cells, and cutaneous infection by the small numbers of bacteria likely to be introduced in this way would promptly be checked by these agencies. In the intestinal mucosa, however, the cholera spirillum finds conditions most favorable for rapid multiplication and the disease is caused by the inflammation and destruction of the mucous and submucous tissues by the poison-

[38] Germ. Plague Com. *Arb. a. d. kais. Gesundheitsamte,* Vol. 16, 1899.
[39] Conte. *Rev. vétérin.,* Vol. 18, 1893.
[40] Galtier. *Compt. rend. de la soc. biol.,* 1890.
[41] Bartel. *Wien. Klinikhandt,* 1906-1907.

ous substances emanating from the large numbers of cholera spirilla which die and are disintegrated, as well as by the absorption of these poisons into the circulation. The bacteria themselves, however, never gain a permanent foothold within the blood or other organs. In the case of typhoid fever the conditions are somewhat similar, although here, during the earlier weeks of the disease, we have an actual penetration of the bacilli into the circulation. This, however, probably takes place only after intraintestinal proliferation has taken place, which then, on the injured mucosa, represents a dose out of all proportion great when compared with the quantities that would spontaneously come into contact with the external surface of the body.

This leads us to another important factor concerning the invading forces, in the determination of successful infection, namely, that of the *quantity introduced or the dosage.*

In order to cause infection, even when the bacteria are of the variety known to produce disease or "pathogenic," and are brought into contact with the body by a path suitable to their peculiar requirements, the initial quantity introduced must be sufficiently large to preclude complete annihilation by the first onslaught of the defensive powers of the body. It is plain, therefore, that in the case of bacteria weak in power to cause disease, given the subject of infection and his defences as a constant, the quantities to be introduced must be larger than in the case of micro-organisms of violent disease-producing properties. The dosage necessary to cause infection, therefore, is in inverse proportion to that property of bacteria spoken of as their *"virulence."* Thus we measure the degree of the so-called virulence of bacteria by determining the smallest quantity, measured by dilution of platinum loops or by fractions of agar slant cultures (both very inexact methods), which will still cause infection and death in susceptible animals of a standard weight. In the case of micro-organisms of extreme virulence, such as the anthrax bacillus or bacilli of the hemorrhagic septicemia group, the inoculation of a very small number of bacteria may suffice to initiate infection. Indeed, it has been claimed for the anthrax bacillus that the injection of a single bacterium will produce fatal disease in a susceptible animal. The inverse relation existing between the degree of virulence and the number of bacteria inoculated is well illustrated by the experiments of Webb, Williams, and Barber,[42] carried out upon white mice with anthrax, by the method of inoculation devised by Barber.[43] This technique consists in picking up single organisms with a capillary pipette under microscopic control, from a very thin emulsion of bacteria and injecting directly from the pipette through a needle puncture in the skin. While requiring a considerable de-

[42] Webb, Williams, and Barber. *Jour. Med. Res.*, 1909, Vol. XV.
[43] Barber. *Kansas Univ. Science Bulletin,* March, 1907.

gree of skill, the method, when successful, permits an actual accurate count of injected bacteria instead of the merely approximate estimate which can be made by consecutive dilutions of thicker emulsions. In their experiments with anthrax in white mice Webb, Williams, and Barber found that the inoculation of a single thread of anthrax bacilli (3 to 6 individuals) taken directly from the blood of a dead animal (that is, in the most virulent condition) would regularly cause death, and it was impossible for this reason to immunize with such bacilli. On the other hand, if taken from 12-hour agar cultures of the same strain such small quantities would often fail to kill. The brief period of growth under artificial conditions had sufficiently lessened the virulence of the bacilli so that 2, 3, and more threads could be injected without harm. And after several generations of such cultivation as many as 27 and more threads could be inoculated with impunity.

Another example of the measurement of relative degrees of virulence, by a method more commonly employed, may be illustrated as follows: The problem in which this particular measurement was used consisted in the comparison of the virulence of two strains of pneumococcus, one (N_2) successively passed through white mice, the other (N_1) kept alive for several weeks on serum-agar. To accomplish this graded quantities of 18-hour broth cultures of the two strains were injected into mice of approximately the same weight, as follows:

N_1	Result	N_2	Result
0.1 c. c.	= dead 24 hrs.	0.1 c. c.	= dead 24 hrs.
0.05 c. c.	= lives	0.05 c. c.	= dead 24 hrs.
0.02 c. c.	= lives	0.02 c. c.	= dead 24 hrs.
0.01 c. c.	= lives	0.01 c. c.	= lives

Accidental Factors Favoring Infection.—In discussing the problems of virulence we must not forget that there are many accidental factors which may make an infection likely where, otherwise, it might not have occurred. Trauma is perhaps the most important of these. It is well known that injury in which tissue is killed will favor the development of streptococci and staphylococci, which in the same dose and with the same degree of virulence entering into wounds incised with a minimum tissue destruction, might have been easily overcome by the rapidly mobilized defences of the body. In tetanus and other anaerobic infections, this is, of course, a well-known state of affairs, and has been again and again proved in connection with war wounds. In these infections, moreover, the additional danger of concomitant infection with other organisms is of importance. If, for instance, staphylococci are in-

jected at the same time with tetanus spores, the chances of the development of tetanus are vastly increased, as has been shown by workers in the United States Hygienic Laboratory. There are certain epidemic infections, too, which are more dangerous in connection with their ability to prepare the way for secondary invasion, than they are in themselves. Most important among these are measles and influenza. Pure influenza is a mild disease, but an influenzal infection of the respiratory tract seems to render the subject susceptible to secondary invasion with streptococci and pneumococci to an extreme degree, a fact which is responsible for by far the greater part of the mortality in influenza epidemics. This is almost equally true of measles, where the disease itself is rarely fatal, but prepares the way for fatal respiratory infection with streptococci and pneumococci, particularly, and perhaps for subsequent tuberculosis. To a less marked degree, the same thing is true of whooping cough where secondary influenza bacillus, pneumococcus and streptococcus invasion may follow very promptly upon the initial Bordet-Gengou infection. A considerable number of analogous examples could be cited.

Types of Infection.—From the facts we have discussed in the preceding paragraphs it now becomes manifest that the elements which determine the nature of an infectious disease are twofold. On the one hand each variety of infectious germs possesses certain biological and chemical attributes which are specific and peculiar to itself; by these its predilection for path of entrance and mode of attack is determined, and upon these depends the nature of the reaction called forth in the animal body. On the other hand the degree of infection in each case, the severity of the reaction and the ultimate outcome are determined by the balance which is struck between the virulence of the entering germ and the protective mechanism opposed to it.

The specific properties of each micro-organism are the factors which account for the clinical uniformity (within definite limits) which is observed in the maladies produced in different individuals by the same species of bacteria. Thus a severe typhoid fever is, in essential characteristics, entirely similar to a mild case—since in both instances the path of entrance, through the intestine, is the same, the distribution of the germs after entrance differs only in degree, and the reactions, local and systemic, which are called forth are alike. And cases of this disease in general differ as a class from the maladies caused by, let us say, the group of clinical conditions resulting from anthrax infection, where entrance is through the skin, and generalized infection of the blood ensues without definite or regular localization in any given organ. Again, a localized staphylococcus abscess will differ materially from an equally localized focus of tuberculosis, because the chemical constituents of these

bacteria respectively call forth each a characteristic response on the part of the defensive mechanism.

Such specificity of the various micro-organisms may of course be due partly to their mode of attack and distribution, and partly, as we shall see, to the pharmacological action of the poisonous products given out by them.

That both factors contribute seems beyond doubt; but recent work, especially that of Friedberger, which is fully discussed in another place indicates the possibility that clinical differences depend much less than was formerly supposed upon specificity of the intracellular poisons, and much more upon distribution and localized accumulation of the germs, conditions which are determined rather by the mode and extent of invasion than by chemical differences of poison production. This problem, rather difficult to discuss on the limited basis of the facts so far outlined, will become clearer as we proceed, but we need only refer at present to the essential clinical uniformity of the various forms of septicemia, where organisms freely circulate in the blood—with often a focus of distribution on a heart valve—conditions in which it is rarely possible to determine the species of the responsible germ except by blood culture. Or, again, as Friedberger [44] points out, there is great similarity between the ordinary pneumococcus pneumonia and that caused by the Friedlander bacillus. In both cases the distribution and mode of attack of the bacteria are essentially the same, though the micro-organisms themselves are biologically very dissimilar.

One and the same micro-organism, on the other hand, may cause entirely different clinical conditions, and here the type of infection depends purely on the degree of invasion possible in the given case—that is, the balance between virulence and resistance. A germ may enter the body and cause an inflammatory reaction at the point of entrance, the process remaining purely localized. In such cases the defensive forces have been so efficient, the invasive properties of the germ so relatively weak, that progression beyond the point of entrance is prevented and the resultant disease takes the form merely of a localized abscess. This is the case when a healthy individual is infected with an attenuated organism or by one whose species' characteristics do not include a powerful invasive property. Thus streptococci, if entering the tissues of a normal subject in small numbers or in attenuated form, may produce a purely localized infection, and ordinarily non-pathogenic germs like proteus, subtilis, or colon bacilli may produce localized abscesses in weak and debilitated individuals, though implanted upon a healthy subject they would be rapidly disposed of without gaining even a preliminary foothold. Such tendency to localization is the common form of infection in the case of a number of germs. It is the most usual type

[44] Friedberger. *Deutsche med. Woch.*, No. 11, 1911.

of staphylococcus infection, for instance, in which the degree of virulence of the strains ordinarily met is such that the balance struck by them with the average defensive powers of man results in localization. However, the same micro-organism, enhanced in virulence, or gaining entrance in unusual numbers in a weakened individual, may rapidly spread from the point of inoculation, at first by contiguity, then by invasion of the blood and lymph channels, and become generalized.

When organisms become generalized and circulate in the blood the resulting condition is spoken of as septicemia or bacteriemia. This is the form of infection commonly caused by streptococci, bacilli of the hemorrhagic septicemia group, anthrax bacilli, and many others. It implies a powerful invasive property and always constitutes a condition of great gravity when persistent. We are learning of recent years, however, that in many infectious diseases formerly regarded as purely localized a temporary entrance of the bacteria into the circulation is a usual occurrence. Thus Fraenkel [45] has shown that lobar pneumonia is almost always accompanied during the acute stages of the disease by pneumococcus septicemia, and in typhoid fever we now know that the organisms circulate freely in the blood during the first two weeks of the disease, and often longer than this.

In these and other conditions the bacteria may be gradually destroyed and disappear from the blood stream as the immunity of the subject increases. In other cases the bacterial activities may be partially checked, the process becoming slower and more chronic. This is especially often the case when micro-organisms after entrance to the circulation have found a secondary lodgment upon a heart valve, from which a continuously renewed supply of bacteria can be given off to the blood. A special form of such "malignant endocarditis" caused by the *Streptococcus viridans* is particularly apt to take this chronic course.

The presence of bacteria in the blood is not, therefore, as formerly supposed, an invariably fatal condition.

Adami's recent work would indicate, moreover, that bacteria may normally enter the portal or even the general circulation from the intestine during health. This condition of "sub-infection," as he calls it, is more fully discussed on p. 256. That colon and other intestinal bacteria may often penetrate into the portal circulation is indicated by the occasional occurrence of colon bacillus abscesses after trauma of the liver. In most septicemias, however, caused by virulent bacteria the invasion of the blood stream persists, rapid multiplication occurs and leads to death.

From the circulation the bacteria may gain lodgment in various organs and cause the formation of secondary abscesses. This condi-

[45] Fraenkel. *V. Leyden Festschr.*, 1902.

tion is known as "pyemia," and may be caused by almost any bacteria which are capable of producing septicemia. Thus staphylococci, streptococci, or pneumococci may lodge in bones, joints, brain, or kidneys, in fact in any organ in which they can gain a foothold. However, there are evidences of distinct tissue predilections on the part of certain germs. Thus the virus of rabies and that of poliomyelitis, though to some extent universally distributed, seem especially to concentrate in the nervous system; cholera spirilla and dysentery bacilli appear to find conditions most favorable for development in the intestinal mucosa; amebic abscesses are most common in the liver; gonococcus infections when generalized find secondary localization with particular frequency on heart valves and joints; leprosy bacilli have a predilection for the nerve sheaths; and glanders bacilli injected into the peritoneum of a male guinea pig localize with such regularity in the testicles that the experiment has diagnostic value (Strauss test). Conversely it is only explicable on the assumption of such selective lodgment that tubercle bacilli, even though otherwise universally distributed through the body, will be absent from striped muscle tissue, and rare in the walls of the stomach. Such selection, as far as we can account for it all, seems to depend upon the varying cultural conditions encountered by the germs in different organs.

Among the most specific and curious of the selections of special tissues is that recently worked out with the probably closely related infectious filtrable agents of herpes, encephalitis and the salivary viruses first described by Levaditi. Into the same general class probably belongs the virus of small-pox and of chicken-pox. These, as yet uncultivated and probably, also, unseen micro-organisms, can be inoculated upon animals, but appear to be limited in their infectious powers to cornea, skin and nervous system, rigidly so in some cases, less so in others, all of these tissues being, in their embryological origin, ectodermal.

On the other hand, localization may also be dependent upon accidental conditions such as trauma. Infections in which the entrance of bacteria is coincident with injury—as in the case, for instance, of compound fractures—will be able to spread throughout the injured region much more easily than they could enter the healthy tissue. In fact, it is well known that local tissue injury at the point of inoculation favors infection since it furnishes a rich substratum for growth in the form of dead cells or blood clot and interferes with the accomplishment of a normal protective reaction. In cases in which bacteria are circulating in the blood mechanical injury may create a focus of reduced resistance on which the invaders can gain a foothold. It is in this way perhaps that, among other things, we can explain tuberculosis of joints or bones which present a history of injury preceding the development of the infec-

tion—or the pleurisy and lobular pneumonias which have been known to ensue upon the fracture of a rib.

It is also possible that bacteria may be distributed in various organs directly from the initial focus by embolism or by the massive invasion of a blood vessel. It is by such breaking into a vein that Weigert explains the generalization of miliary tuberculosis.

The inflammatory reaction which usually ensues at the point of entrance of bacteria is merely a result of the local struggle between invader and tissues, and the violence of this reaction is in a large measure an indication of the resistance of the infected subject. When, for instance, a streptococcus of moderate virulence gains lodgment in the skin of a healthy individual the rapid mobilization of leukocytic and other defences may prevent further invasion by the bacteria and lead to a struggle which is clinically evidenced by severe local symptoms. Did the virulence of the streptococci far overbalance the powers of resistance the local struggle might be reduced to a minimum, the infection progressing without any, or with but a slight local, reaction. The fact that pneumococci lodging in the human lung ordinarily cause lobar pneumonia is merely an evidence of a considerable degree of resistance to these germs on the part of the average human being. Pneumococci introduced into the pulmonary alveoli of very susceptible animals (rabbits) may pass directly through into the circulation, causing fatal septicemia without leading to a more than mild and temporary reaction in the lungs themselves. If, as in Wadsworth's [46] experiments, the rabbits are partially immunized—that is, their resistance increased before the pulmonary inoculation is carried out—a violent local reaction, analogous to lobar pneumonia, may follow, the severity of the reaction at the portal of entry being manifestly an evidence of more energetic opposition to further penetration of the bacteria.

The entrance of bacteria into the deeper tissues, and even the circulation, without any, or with but slight, local evidences of infection at the point of entrance is by no means rare. The innocent appearance of the site of the entrance of the bacteria in generalized streptococcus infection is a common surgical observation, and a streptococcus-infected wound of the hand or leg in a patient dying of septicemia may appear but slightly inflamed and edematous and incomparably milder in appearance than a staphylococcus boil with which the patient is walking about and suffering hardly any systemic disturbance.

Incubation Time.—Between the time of entrance of the bacteria into the body and the first appearance of symptoms of disease there is always a definite interval which is spoken of as "incubation time." This period is made up of two definite divisions—one the time necessary for growth, distribution, and accumulation of the bacteria,

[46] Wadsworth. *Am. Jour. of the Med. Sc.,* Vol. 27, 1904.

the other the time necessary for the action of the toxin or poison which may be secreted. The latter, the incubation time of the toxin, is a subject which is still unclear in many of its phases, and will be discussed in the following chapter (see p. 43). The former, however, is easily comprehended, in fact, is to be expected. For the small number of bacteria which gain entrance to the tissues in spontaneous infection is entirely inadequate in itself to produce symptoms. It is necessary that multiplication shall take place until the bacteria have accumulated in number sufficient to cause noticeable physiological disturbance. That the interval necessary for this must vary according to the number of bacteria originally introduced, the virulence of these, and the specific resistance of the patient goes without saying. Von Pirquet and Schick have suggested also that the incubation time may correspond roughly to the interval during which the subject is becoming "allergic" or hypersusceptible to the bacteria or virus. This will be discussed at greater length in the chapter on anaphylaxis.[47]

But within the limits of the variations introduced by these factors the incubation time of each infectious disease—if spontaneously acquired—is sufficiently uniform to be characteristic. Thus the primary lesion in syphilis follows the inoculation after an interval of two or three weeks, rabies follows inoculation with street virus after about four to six weeks, the period being somewhat dependent on the location of the bite; typhoid fever takes about two weeks to develop; gonorrhea about five to seven days; small-pox about two weeks; yellow fever three to five days; and scarlet fever and diphtheria about two to six days. In general, it may be stated that within the limits observed for each particular infection the shorter the incubation time the more severe is the infection. Thus if tetanus follows inoculation with the tetanus bacillus within seven days the prognosis is far more grave than when the incubation time has occupied two or three weeks. And if localized and general symptoms follow rapidly (within twenty-four to forty-eight hours) after a streptococcus infection it is likely that the process is a very severe and virulent one.

Effects of Symbiosis upon Infection.—That the influence of various species of bacteria growing in the same culture is of great mutual importance to the growth and life of the individual species is, of course, well known to bacteriologists. Such interdependence may be noticeable as antagonism, usually due to inhibition of one species by the metabolic products of the other. On the other hand, even in culture, two different species may enhance each other's activity. This has been particularly noticed with diphtheria bacilli and streptococci in culture by Hilbert.[48] While the condition of

[47] Von Pirquet u. Schick. *Wien. kl. Woch.*, 16, 1903, pp. 758 and 1244.
[48] Hilbert. *Zeit. f. Hyg.*, 29, 1895.

mutual enhancement in culture is rare and perhaps questionable, even in the cases described, in infections of the body it is common and logically so, since one micro-organism may be able to ward off the defensive mechanism of the body from the other, or by its own destructive processes, create conditions more favorable to the development of the offences of its mates. Examples of such symbiotic enhancement of virulence are the association of diphtheria bacilli and streptococci in the throat, and a similar association of the organisms of Vincent's Angina with diphtheria; in both of these cases the clinical diphtheria is apt to be considerably more severe than when the diphtheria bacillus is found alone with the ordinary inhabitants of the nose and throat. An extremely important example is that which has been much investigated, in connection with tetanus infection. Here it has been shown that the destructive effects upon tissues exerted by simultaneous staphylococcus or streptococcus infections in the wound, may be instrumental in creating the conditions that favor the development of tetanus. The association of streptococci with scarlet fever is not so clear an example since there is still a possibility that the streptococci may be more intimately related to the disease than merely as symbiotic accompaniments. Upon this point no opinion is, as yet, entirely justified. Of the greatest epidemiological importance in this connection is the incontrovertible fact that diseases such as influenza and measles pave the way for severe and fatal invasion of the respiratory tract and lungs with streptococci and pneumococci; in fact, so much is this the case that the original diseases, themselves, rarely kill, but have a high death rate owing to the secondary invaders for which they prepare the field. Of probably similar significance, also, are the conditions prevailing in hog cholera and in typhus fever where, in the former case, the so-called hog cholera bacillus is almost always associated with the disease, although we know that the specific etiological agent is quite a different organism, a filtrable virus. In typhus fever the frequent presence of the Plotz bacillus and other organisms may represent a similar state of affairs. Thus, while we know very little about the mechanism in most cases, the concomitant presence of a number of infectious agents may very severely increase the ability of either or of both, to invade.

Presence of Bacteria in Tissues in a Latent Condition.—A very interesting fact connected with the reactions between bacteria and tissues is the occasional local balance struck between infectious agents and the tissues in which they lie. In such diseases as tuberculosis and syphilis this phenomenon is quite common. The organisms may remain in a definite place for a month and even longer, without giving rise to any signs of disease, and yet, at a given moment, often without apparent cause, a characteristic inflammatory process may be initiated. In the case of experimental

syphilis, we have observed a rabbit inoculated with virulent treponema in the testis which showed absolutely no signs of reaction for three and one-half months, at which time a typical process began to appear. The phenomenon has been observed with many different bacteria, even with those ordinarily causing acute disease. Not long ago we saw a patient that was extensively incised in the course of a hemolytic streptococcus infection of the hand. A second operation made for purposes of improving function at a time when no inflammation whatever existed in the part, revealed the presence of the same hemolytic cocci which had apparently remained latent in the tissues for the entire interval. Many instances of the same phenomenon may be cited, and on the basis of these, it is quite easy to understand how trauma or other accidents may occasionally give rise to what we speak of as cryptogenic infections.

CHAPTER II

BACTERIAL POISONS

When bacteria have gained a foothold anywhere within the animal body the local and general disturbances which follow, in all but the mildest and most trifling cases, are such that we cannot account for them solely on the basis of mechanical injury.

It may well be that the obstruction of capillaries and lymphatics and the pressure upon parenchyma cells, always incident to inflammatory reactions, contribute materially to local destruction, and thereby indirectly to systemic effects. However, even in diseases like anthrax, in which the body of the victim after death is found flooded throughout with masses of bacteria, these factors cannot fully explain the clinical manifestations. And such cases, indeed, are extreme examples, since, in the large majority of bacterial diseases, the illness resulting in the patient is severe out of all proportion to the extent of the tissue area invaded.

Moreover, all infections, if at all severe, whatever their nature or localization, give rise to fever, and this symptom alone, if carefully observed from hour to hour, may be sufficiently characteristic to indicate the specific micro-organism which is causing the illness. With this there occur alterations of the blood picture, either a numerical increase of white blood cells (leukocytosis) or a change in the relative proportions of the different kinds of leukocytes—or again an anemia caused by the destruction of red cells. There may also be degenerative changes in parenchyma cells of organs far removed from the actual site of bacterial lodgment. All these facts indicate very definitely that, apart from localized tissue destruction or purely mechanical interference with function by capillary obstruction or pressure, there is at the same time an absorption of poisonous substances emanating from the bacteria.

Ptomains.—From the earliest days of logical investigation into the nature of infectious disease, as soon, in fact, as cultural methods had been introduced, bacteria were studied with the purpose of throwing light upon this phase of their activity. As a result of such investigations Selmi,[1] in 1885, described certain basic toxic tubstances which he obtained from putrefying human cadavers and for which he suggested the designation *"ptomain"* (from πτῶμα = dead body). These poisons were later more extensively studied by

[1] Selmi. Cited from Hammarsten, "Textbook of Physiol. Chem.," p. 16.

Brieger,[2] Gautier,[3] Griffiths,[4] and others, and it was at first surmised that the formation of such substances in the infected animal might be held responsible for the toxemic manifestations which accompany bacterial disease.[5]

This, as we shall see, is not the case. Ptomains are probably not formed in traceable quantity in the living tissues and are not in any way identical with the specific bacterial poisons which are responsible for the toxemia of infectious diseases. Nevertheless, they have some pathogenic significance, since they are invariably products of the proteolysis caused by bacteria and can give rise to illness when ingested with putrefying foodstuffs. It is important, therefore, that we discuss them briefly and consider their fundamental distinction from the true bacterial poisons.

Whenever dead organic material, meat, fish, vegetable refuse, etc., is left to itself under suitable conditions of moisture and temperature, putrefaction sets in. As a result of bacterial growth the protein is broken up and among the intermediate products of such proteolysis ptomains appear. Chemically [6] these substances are basic nitrogenous compounds which may or may not contain oxygen. Because of their basic and often highly toxic properties they have been spoken of as "animal alkaloids." Many of them contain only C, H, and N, and are ammonia substitution products. (See Vaughan and Novy, *loc. cit.*, p. 248.) Thus some of the simpler ones are:

Methylamin$=(CH_3)\ NH_2$
Dimethylamin$=(CH_3)_2\ NH$
Trimethylamin$=(CH_3)_3\ N$

Among those somewhat more complex are:

Putrescin$=NH_2-CH_2-CH_2-CH_2-CH_2-NH_2$
and Cadaverin$=NH_2-CH_2-CH_2-CH_2-CH_2-CH_2-NH_2$

Samuely classifies the ptomains according to their nitrogen contents as follows:

1. Those with one nitrogen atom $(C_8H_{11}N)$ $(C_8H_{13}N)$ $(C_{10}H_{15}N)$

2. Those with two nitrogen atoms such as putrescin $(C_4H_{12}N_2)$ and cadaverin $(C_5H_{14}N_2)$ and

[2] Brieger. "Die Ptomaine," Berlin, 1885; *Virchow's Archiv.*, Vols. 112 and 115; *Berl. klin. Woch.*, 1887, 1888.

[3] Gautier. Cited after Pick, *Bull. de l'acad. de méd.*, 1886.

[4] Griffiths. *Compt. Rend. de l'acad. des sc.*, Vol. 113.

[5] For a historical outline of our knowledge of these poisons, as well as for a thorough treatment of their nature, see Vaughan and Novy, "Cellular Toxins."

[6] For a discussion of the chemistry of the ptomains see Vaughan and Novy, "Cellular Toxins," Lea Bros., Philadelphia, 1902. See also Samuely in Oppenheimer's "Handbuch der Biochemie," Vol. I, pp. 794 *et seq.;* and Wells', "Chemical Pathology," Saunders, Philadelphia, 1907.

3. Those with three nitrogen atoms such as methyl guanidin ($C_2H_7N_3$).

4. Finally there is an important group which contains oxygen, such as the substance sepsin ($C_5H_{14}N_2O_2$) obtained by Faust from putrefying yeast cells.

They are not in all cases protein cleavage products, since bodies of the cholin group, cholin, neurin, and muscarin, the two last named highly toxic, are lecithin derivatives, and Samuely points out that other lipoid cleavage products, always present in decomposing tissues, may well contribute to ptomain production in the presence of a source of nitrogen. It is interesting to note also that the vegetable poison muscarin, isolated by Schmiedeberg from mushrooms, is chemically identical with a toxic base found by Brieger in decomposing fish.

The ptomains are not poisonous in every case. The chemically simpler ones like methylamin, di- and trimethylamin possess little or no toxicity. Others chemically more complex—like cadaverin and putrescin—may be capable merely of causing local necrosis, while sepsin, closely related to cadaverin in chemical constitution, but containing oxygen, is a powerful poison which acts violently upon the intestinal blood vessels, causing capillary dilatation, congestion, and diapedesis.[7] The presence of oxygen seems indeed to be necessary for the development of strong toxicity (Brieger, Vaughan, and Novy). Again, the lecithin derivative, cholin, is but weakly toxic, while neurin is exceedingly poisonous. In putrefying mixtures these toxic bodies appear on or about the fifth or seventh day after putrefaction sets in, and disappear, by further cleavage, more or less rapidly, yielding less complex nitrogenous substances that are non-toxic.

With the limited knowledge regarding bacteria and infectious diseases at the disposal of the earlier investigators it was but natural that the discovery of ptomains in cultures of putrefactive bacteria aroused the suspicion that these bodies were responsible for the toxemia of infectious disease.

The search for poisonous substances in pure cultures of pathogenic bacteria was, therefore, assiduously taken up by Brieger and his pupils, and, in truth, ptomains were actually found as products of some of the disease-producing micro-organisms, just as they had been found in the mixed cultures involved in the putrefaction of meat. Thus cadaverin was found in cultures of the cholera spirillum, another nitrogenous poison, typhotoxin, in those of typhoid bacilli, and still another in tetanus cultures, all of them producing more or less severe illness when injected into animals.

In spite of this evidence, however, we have been forced to conclude that the ptomains cannot properly be held responsible for bac-

[7] Meyer and Gottlieb. "Experim. Pharmakologie," 2d ed., p. 262.

terial toxemia as manifested in disease. In the first place it is doubtful whether ptomains, in noticeable quantity, are ever produced within the living infected body. Then, again, potent ptomains are produced in culture by many bacteria having absolutely no pathogenic power, while highly pathogenic bacteria may produce little or no ptomains. Ptomain production, moreover, is not specific, since the same ptomains may be produced by many different bacteria or mixtures of bacteria, provided the conditions of nutrient materials and temperature are favorable for growth. We cannot therefore account for bacterial toxemia, in which the poison produced by an individual species is characteristic and invariably the same, under varying cultural and environmental conditions, by the production of ptomains. And even when ptomains are produced in culture fluids by pathogenic bacteria their physiological action is usually quite different from that of the poisons produced by the same micro-organisms in the infected subject.

Briefly summarized, therefore, the ptomains are poisons elaborated by all bacteria that are capable of producing protein cleavage, if planted on suitable nutrient materials under conditions favoring growth. The matrix of these poisons is the protein nutriment; they are not products of intracellular metabolism specifically characteristic of the bacteria which produce them.

Their importance in the production of disease, therefore, is really an indirect one. They may cause disease if putrid meat or other material is ingested, and with it preformed ptomains, which may be taken in and further elaborated by continued putrefaction in the intestines. This form of meat poisoning, without bacteriological investigation, may be difficult to distinguish from such bacterial forms of meat poisoning as those caused by the Gärtner bacillus or the bacillus botulinus. Novy [8] believes that true ptomain poisoning of this kind is rather less frequent than formerly supposed. However, in such cases as those of Vaughan, who isolated a poisonous ptomain "tyrotoxicon" from cheese and milk, their importance seems reasonably certain. It is also probable that certain forms of autointoxication may be caused by the production in the intestinal canal of ptomains resulting from bacterial putrefaction incident to faulty digestive conditions. It is the antagonism to such intestinal putrefaction by the acid production of the bacillus Bulgaricus which is probably the basic cause of any favorable therapeutic effects which have attended the soured milk therapy of Metchnikoff. Again the growth of saprophytes in necrotic tissues such as gangrenous extremities in diabetes or amputation stumps, may lead to the formation of ptomains which, after absorption, can cause disease. In all such cases the process is one determined by the bacterial putrefaction of dead organic materials, and the absorbed

[8] Novy in Osler's "Modern Medicine," Vol. 1, p. 223.

poisons are not true bacterial toxins, since they do not emanate specifically from the cell substance of the micro-organisms but rather represent incidental cleavage products of the nutrient materials. Therefore, also, the ptomains are unspecific—their formation a common attribute of a large variety of saprophytic organisms, their production, as to quantity and kind, primarily dependent upon the nature of the nutrient materials on which the bacteria are grown.

Other Non-Specific Toxic Effects.—The fermentative and putrefactive activities of many bacteria not ordinarily classified as pathogenic, may give rise on occasion to disease if they take place in the intestinal canal by virtue of a temporary or chronic alteration of the flora normal to this location. It is well known, of course, since the studies of Escherich,[9] Herter,[10] Kendall,[11] Rettger,[12] and others that the infant bowel, sterile at birth, rapidly begins to harbor bacteria. For the first few days there is a period of irregular accidental flora which is soon transformed into a characteristic infantile intestinal flora, changing only as diet changes in the course of years. In breast-fed children, the upper part of the small intestine, for instance, will usually contain various Gram positive cocci which predominate over the Gram negative bacilli. Further down, organisms of the Colon, Lactis aerogenes type appear, and in the lower part of the cecum, Gram positive bacilli, like the Bifidus of Tissier and other anaerobes predominate. As breast feeding is discontinued, the flora changes by the superimposition of Colon bacilli and the acidophilic group. For the purpose of making our point it is not necessary to go into details concerning the actual flora, for which we refer the reader to the original Monograph and to a brief summary in our own "Textbook of Bacteriology." It is sufficient to indicate that the ingestion at any time of milk or other food, very highly contaminated with organisms capable of powerful fermentative action upon carbohydrates with the formation of various acids and their by-products, or with masses of putrefactive organisms capable of splitting proteins to various degrees, may give rise, on the one hand, to the absorption of many abnormal cleavage products of food materials, considerable amounts of moderately toxic bacterial products and, in addition to this, interfere with digestion and the normal movement of the intestinal wall by distention with large volumes of gas. In this sense, not only ptomains but perhaps acid products of carbohydrates, digestion, etc.,

[9] Escherich. "Darmbakterien des Saüglings," Stuttgart, 1886.

[10] Herter. "The Common Bacterial Infections of the Digestive Tract," Harvey Lecture, 1906-1907.

[11] Kendall. "Bacteriology, General, Pathological and Intestinal," Lea & Febiger, Philadelphia, 1916, p. 580.

[12] Rettger. *Jour. Biochem.*, 2, 1906.

may readily give rise to intoxication, constipation or diarrheal disease, or, as is often the case, constipation followed by violent diarrhea as the stagnated material is subjected to further bacterial action. It is this form of non-specific disease production on the part of many bacteria ordinarily not considered pathogens, which is perhaps most neglected and overlooked in clinical work. Judging from the work of Park, Holt and their co-workers, upon infantile diarrhea in relation to heavily contaminated milk, this mechanism may play an important rôle in the summer mortality of children.

Specific Bacterial Poisons.—In contradistinction to the ptomains, the specific bacterial poisons, in the technical meaning of the term, are substances which are characteristic for each individual species of bacteria and truly the products of bacterial metabolism in that they emanate from the cell itself, either as a secretion or excretion during cell life, or as an inherent element of the cytoplasm liberated after death (or possibly as a cleavage product of the disintegrating bacterial protein).[13] They are dependent upon the nature of the culture medium only in so far as this favors or retards the normal development of the micro-organisms. While, therefore, a diphtheria bacillus undoubtedly produces the largest quantities of its specific poison on bouillon suitably prepared for this particular purpose, it will also, in smaller amount, produce qualitatively the same poison on all media on which its growth is free and uninhibited, even on a medium such as that of Uschinsky, which is entirely devoid of proteins. The *toxins* are, therefore, elements of intracellular metabolism, permanently or transiently constituent parts of the cell body.

A specific bacterial toxin was first obtained from the diphtheria bacillus by Roux and Yersin[14] in 1889. They discovered that if diphtheria bacilli were grown on veal broth and the cultures filtered through porcelain candles, after seven days at 37.5° C. the filtrates were highly toxic, producing the same symptoms and autopsy findings in rabbits, guinea pigs and birds which followed the injection of the living bacilli themselves. The poison was therefore a soluble product of the bacteria during the period of their vigorous growth, apparently given up by them to the culture fluid. Very soon after this, in 1891, Kitasato[15] discovered a similar specific toxin in culture filtrates of the tetanus bacillus, and it was the hope of bacteriologists that analogous poisons could be determined for all pathogenic bacteria.

This hope, however, has been disappointed. It was soon found that cultures of cholera spirilla, typhoid bacilli, and many other

[13] In connection with this read the discussion on anaphylaxis in chapter XVII.

[14] Roux and Yersin. *Ann. de l'Inst. Pasteur,* Vol. 2, 1889.

[15] Kitasato. *Zeitschr. f. Hyg.,* 1891, Vol. 10.

germs did not yield toxic filtrates of this kind but that the poisons in these cases seemed to be firmly bound to the bacterial bodies during life, and given up to the surrounding media only after death and disintegration of the cells.

Pfeiffer [16] was the first one to formulate this conception in his studies upon cholera poisons. He found that when cholera spirilla were grown upon broth and filtered after 6 or 7 days, the filtrate was but slightly toxic, but that, in this case, unlike the conditions prevailing in diphtheria and tetanus cultures, the residue of bacterial cell bodies, even after they had been killed by chloroform, thymol, or drying, were powerfully poisonous.

We have then two main classes of specific bacterial poisons. One —typified by diphtheria and tetanus poisons—is produced during the period of energetic growth by the living bacteria, is given off to the surrounding culture fluid as a secretion or excretion, and can be obtained in bacteria-free filtrates at a time when few, if any, of the micro-organisms have died or disintegrated. These are spoken of as "true toxins" or "exotoxins."

The other group—typified by the cholera poisons as described by Pfeiffer—is apparently an intracellular, constituent part of the bacterial body—not given off during life and not, therefore, obtained in filtrates of young living cultures. If the cultures are preserved until cell death has taken place and the dead bodies have been extracted by the culture fluid, the filtrate becomes gradually more toxic. The bodies of such bacteria are in themselves powerfully toxic when injected, dead or alive. These poisons for obvious reasons Pfeiffer has named the *"endotoxins,"* since he regarded them as specific and definite substances, present as such in the living bacterial cell.

In addition to the endotoxins the bacterial protein contains substances which attract and lead to the accumulation of leukocytes. In other words, they exert a positive chemotactic influence. This was first observed in 1884 by Leber,[17] who induced the formation of pus by injecting dead staphylococcus cultures, and, later, found that the same effect resulted from the injection of alcoholic extracts of staphylococci. These chemotaxis-inducing substances were later particularly studied by Buchner. Buchner [18] extracted them from many varieties of bacteria, independent of pathogenicity. Although there are quantitative differences, all bacteria seem to contain such substances, and Buchner believed the chemotactic property to be a general attribute of the bacterial protoplasm. He speaks of his extracts as *bacterial proteins.*

Exotoxins or True Toxins.—The true *toxins* or *exotoxins,* then, appear to be products of living bacteria given off from these very

[16] Pfeiffer. *Zeitschr. f. Hyg.,* Vol. II, 1892.
[17] Leber. "Uber die Entzündung," Leipzig, 1884.
[18] Buchner. *Berl. klin. Woch.,* 1890.

much as are the ferments and enzymes by which micro-organisms cause cleavage of carbohydrates or proteins—and indeed the French school, from the first, compared these toxins to enzymes, with which, as we shall see, they have much in common. The *endotoxins*—on the other hand—at least as conceived by Pfeiffer, are structural ingredients of the bacterial protoplasm which are toxic when brought into solution as the cells break up.

Concerning the accuracy of this conception, however, much doubt has recently arisen, as a result of researches which will be discussed below.

These two types of poison, moreover, differ from each other not only in mode of origin but in biological characteristics far more fundamental than this.

The discovery of diphtheria toxin by Roux and Yersin was followed by diligent investigations into the toxic properties of all known pathogenic bacteria, and it was soon found that a few only of these germs could produce poisons biologically similar to that found in diphtheria cultures. It was in the course of investigations of this kind, indeed, that Pfeiffer, failing to discover an exotoxin in cultures of cholera and other germs, formulated his endotoxin theory.

The list of true toxin or exotoxin producers, then, is short. Among the more important are, in addition to the diphtheria and tetanus bacilli—which have been mentioned above—the *Bacillus botulinus,*[19] the *Bacillus pyocyaneus,*[20] and that of symptomatic anthrax.[21] It has also been claimed that similar toxins are formed by the cholera spirillum (Brau and Denier),[22] by the dysentery bacillus of the Shiga-Kruse type (Kraus and Doerr)[23] and the *Bacillus typhosus* (Arima).[24] In the cases of the three last-named organisms, however, the secretion of a true exotoxin has not been accepted as a fact by all observers. Indeed, even though such substances may possibly be produced by these bacteria in small amounts it is not likely, in the light of our present knowledge, that they play more than a secondary rôle in the toxemic manifestations of cholera, dysentery, and typhoid, the important poisons in these cases being those derived from the bacterial cell bodies.

Similar in essential properties to the true exotoxins also are the erythrocyte poisons (hemotoxins) produced by many bacteria which cause hemolysis of red cells, and the leukocyte-destroying poison (leukocydin) which is a product of the *Staphylococcus aureus.*

All of these "true bacterial toxins" or exotoxins, apart from sim-

[19] Kempner. *Zeitschr. f. Hyg.,* Vol. 26, 1897.
[20] Wassermann. *Zeitschr. f. Hyg.,* Vol. 22, 1896.
[21] Grassberger and Schattenfroh. *Wien Deuticke,* 1904.
[22] Brau and Denier. *Ann. de l'Inst. Past.,* Vol. 20, 1906.
[23] Kraus and Doerr. *Wien kl. Woch.,* 42, 1905.
[24] Arima. *Centralbl. f. Bakt.,* I, Vol. 63, 1912.

ilarity of origin, as soluble secretions of the living bacteria, possess certain common biological characteristics which sharply differentiate them from the "endotoxins." These characteristics they share with a number of non-bacterial substances such as the vegetable poisons ricin, crotin, and abrin, with animal poisons like snake venom and spider poison (arachnolysin), and, in certain important respects, with the substances spoken of as enzymes.

Thus the bacterial true toxins are not biologically unique substances. Both in themselves and in regard to the reactions they elicit when injected into the animal body, they share certain cardinal properties with analogous substances derived from the higher plants and from animals. And it is important to recognize at once that we are dealing here, as in other phases of the study of bacterial immunity, with broad biological laws, which find application not only in bacteriology, but in general pathology and in the phenomena of protein metabolism in general. It so happens that these phenomena have been studied and are most easily elucidated in connection with bacteria. But their general significance must not be lost sight of.

The cardinal characteristic which unites all of these substances into a single well-defined biological group is their property of inducing the formation of *antitoxins* when injected into animals. This property is so important and its thorough comprehension so essential that we may be permitted to digress briefly in order to make it clear.[25]

As we shall see, in subsequent chapters, all substances which lead to the formation of specifically reacting antibodies in the treated animal are spoken of as *"antigens"* or "antibody-inducing substances." The class of "antigens" is a large one, including all known proteins, and possibly some of the higher protein split products, and protein-lipoid combinations, though the "antigenic" properties of the last two are still in controversy. But among this large group of substances it is only the bacterial true toxins (exotoxins), obtained in broth filtrates of living cultures, together with the vegetable poisons and other substances we have classified with them above, which induce in the blood of the treated animal a neutralizing antibody—(antitoxin)—which inhibits quantity for quantity the activity of the injected toxin or vegetable or animal poison. *This property of eliciting the production of antitoxin in the animal body alone separates these substances sharply from all other*

[25] We suggested in a Harvey Lecture some years ago that the differentiation between the exotoxins, enzymes, snake venoms, ricin, abrin, etc., by reason of their production of a neutralizing antitoxin antibody in the treated animal, was such a sharp one from the general protein antigens derived from bacteria and other sources, that it might add considerably to clearness if we separated them in a class under the name of antitoxinogens. This would seem one of the few instances in which it might be desirable to add a new term to the nomenclature of immunology.

antigens, toxic or otherwise, and, in this respect, they differ sharply from the so-called "endotoxins" against which no antitoxins can be produced.

As an important secondary characteristic of this group of substances we may regard their chemically indefinable nature. In the case of none of them have we any definite knowledge of chemical constitution except in so far as it has been hitherto impossible to separate them from the protein molecule. The intensive chemical study of the toxins has universally resulted in failure to obtain a protein-free product which has the characteristic toxic properties of the original filtrate, or its antitoxin-inducing power. Concerning the methods which have been employed in the study of the chemistry of these substances we will have more to say in another place.[26] It is safe to summarize all this work for our present purposes, by stating that, whatever the method employed, until now all of the preparations obtained have given one or another of the protein type-reactions, and that none of them can be positively accepted as protein-free. The results here obtained have been entirely analogous to those obtained in similar investigations upon enzymes. (See also discussion of antigens, chapter IV.)

The analogy with enzymes is indeed a striking one and noted by the first investigators of a true toxin, Roux and Yersin. Biologically, of course, we have the cardinal similarity in that the injection of toxins into animals induces the production of antitoxin, and treatment with enzymes induces specific and neutralizing anti-enzymes. In addition to this, they are alike in their susceptibility to heat (both being destroyed when in solution by temperatures over 80° C.), in their gradual deterioration on standing, and their mysterious activity in small quantities upon disproportionately larger masses of the substances they attack. There is, however, one important difference between the two in their mode of action. For, while the toxins are apparently bound or neutralized by the tissues they attack, the action of an enzyme seems rather to be a process in which the enzyme unites with the substance it acts upon, is released as the result is attained, and freed for further action, without noticeable loss of quantity. Such catalytic properties have not yet been satisfactorily demonstrated for the bacterial toxins. However, there are other modifying factors which may account for lack of similarity in this respect, and in all other important points the two classes of substances are closely analogous.

The property of heat sensitiveness, which is a characteristic of bacterial exotoxins and enzymes, is shared with them by all of the substances mentioned above except snake venoms. Snake venoms are not destroyed completely until the temperature is raised to 75° to

[26] An extensive and authoritative summary of this phase of the subject is that of E. Pick in "Kolle u. Wassermann Handbuch," etc., 2d ed., Vol. 1.

80° C. The earlier contention of Leclainche and Vallée, that the toxin of symptomatic anthrax possessed similar heat stability has been satisfactorily refuted by Grassberger and Schattenfroh,[27] who find that heating it to 50° C. for an hour completely destroys it.

There is another important attribute of the true toxin which deserves discussion, though we are by no means in a position to offer any satisfactory explanation for it. We refer to the *incubation time* which elapses between the administration of a toxin and the occurrence of symptoms. Here again snake poisons form an exception—since local manifestations may appear within an extremely short period after the injection of the venom or as the result of a snake bite. Such absence of incubation time, also, seems to be true of the toxin of the Vibrion Septique by which guinea pigs and rabbits are killed by moderate doses almost without any latent period, whatever. However, in the case of all other toxins there is a definite lapse of time between the entrance of the poison and the first symptoms, local or general. This interval is longer when small doses are given—shorter when the doses are large—but is never entirely eliminated—even when many times the fatal dose is given.

In the case of tetanus poison, for instance, injections into a horse may not cause symptoms for as long as four or five days. In mice, animals that are extremely susceptible, the incubation time may be shortened from 36 to 12 hours if we inject 3,600 lethal doses, but, in any case, whatever the dose, this interval cannot be shortened below 8 or 9 hours.[28] Many attempts have been made to explain this. Ehrlich, as we shall see, assumes that the action of a poison depends upon two occurrences: one, the union of the poison with the vulnerable cell, the other the gradual injury of the cell by the toxic atom groups in the poison molecule. The time necessary for the institution of this process, he believes, explains the interval. Richet has suggested that the toxin itself may not be potent until acted upon by the body of the recipient and transformed into a potent form. His views are more directly related to the phenomenon of anaphylaxis and are discussed in another section. De Waele has recently advanced a theory which implies that the incubation time represents the period necessary for the gradual concentration of the poisons in the vulnerable tissues, a process which depends either upon chemical affinities or solubility of the toxins in the cell lipoids. A little at a time would then be absorbed by the vulnerable cells as they come in contact with the poison, through the circulation, and the symptoms would not appear until a definite intracellular concentration had been attained. His views are so closely bound up with the theories on the selective action of the toxins upon individual

[27] Grassberger and Schattenfroh. "Uber das Rauschbrandgift, etc.," Wien. Deuticke, 1904.

[28] De Waele. *Zeitschr. f. Imm.*, Vol. 4, 1910.

tissues and organs that they will be rendered clear as we proceed with a discussion of the latter.

An important point to remember is that there are only a limited number of bacteria which form true toxins, that is, poisons like the ones described which cause antitoxin formation. The most important of these are: Diphtheria bacillus (Behring and Wernicke), Tetanus bacillus (Behring and Kitasato), B. Chauvii, or Bacillus of Symptomatic Anthrax (Grassberger and Schattenfroh),[29] Bacillus botulinus (Kempner),[30] Bacillus pyocyaneous (Wassermann),[31] Welch bacillus (Bull and Pritchett),[32] Vibrion septique (Robertson),[33] Bacillus oedematiens (Weinberg and Seguin).[34] Toxin formation has been claimed, and is likely for the Shiga type of dysentery bacillus.[35] Toxin production has been claimed, but is doubtful in the case of the cholera spirillum, the typhoid bacillus and a number of other organisms.

Within the limits of our definition of true toxins of bacterial origin, also are the leucocidins of the Staphylococcus aureus and the hemolysins of the streptococci, staphylococci and some other bacteria.

Toxic Effects in the Cases of Bacteria which do not form True Exotoxins.—The majority of pathogenic bacteria do not, as we have seen, produce *true toxins* or *exotoxins.* Cultures of cholera spirilla, plague bacilli, and of many other bacteria do not yield toxic filtrates until the cultures have been allowed to stand for prolonged periods during which extraction and possibly autolysis have occurred. In these cases, moreover, definite toxic properties can be demonstrated in the dead cell bodies or in extracts prepared by various methods. In no case, however, is the injection of these "endotoxins" followed by the production of antitoxins. It was very natural to suppose that in micro-organisms of this class the toxic principle might be present in the form of a preformed intracellular poison which could be extracted or which became free as cell-death occurred and disintegration ensued.

It was assumed that, when bacteria entered the animal body and were destroyed by the action of the serum or cells, these endotoxins were liberated and poisoning resulted. The very protective action of the serum, which prevented the extension of the infectious invasion, by limiting bacterial growth, was thus looked upon as the agency by which the endotoxins, toxalbumins, were set free. Experiments by Radziewsky and others, in which it was shown that large

[29] Grassberger and Schattenfroh. *Munch. med. Woch.,* 1900 and 1901.
[30] Kempner. *Zeit. f. Hyg.,* 26, 1897.
[31] Wassermann. *Zeit. f. Hyg.,* 22, 1896.
[32] Bull and Pritchett. *Jour. Exper. Med.,* 26, 1917, 876.
[33] Robertson. *Jour. Pathol. and Bacter.,* 1920.
[34] Weinberg and Seguin. *La Gangrene Gazeuse,* Masson, Paris, 1920.
[35] Todd. *Brit. Med. Jour.,* 2, 1903, 1456; Olitsky and Kligler. *Jour. Exper. Med.,* 31, 1920, 19.

doses of bacteria injected into immunized animals were violently toxic and more rapidly fatal than corresponding amounts injected into normal animals, were taken to mean that in the immune animals a more powerfully bacteriolytic property of the serum led to a more rapid liberation of the endotoxins.

This was the conception of Pfeiffer and, in more recent theoretical discussions, that of Wolff-Eisner. Its essential features consisted in the assumption that the poisons were preformed and were contained within the cell body as such, and that they were specific for each micro-organism, determining to a certain extent its pathogenic properties. Thus typhoid endotoxin, cholera endotoxin, or dysentery endotoxin was supposed each to possess its own particular pharmacological properties by which the clinical manifestations of the respective diseases were partially determined.

It is chiefly the work of Vaughan [36] which has begun to throw doubt upon Pfeiffer's original views, in that Vaughan has shown that all proteins, bacterial or otherwise, would yield, upon cleavage with alkalinized alcohol, toxic split products which possessed many of the pharmacological properties of the so-called endotoxins. In fact, Vaughan succeeded in producing, in animals, fever and other symptoms which are generally associated with infection, merely by injecting into them graded quantities of toxic split products obtained from bacterial protein.

Following Vaughan, Friedberger succeeded in showing that toxic substances similar to Vaughan's split products are formed when bacteria of various species are subjected to the action of normal or immune sera, and that such poisons were pharmacologically alike and produced with equal ease from pathogenic and non-pathogenic micro-organisms. These phenomena are discussed in greater detail in our section on bacterial anaphylaxis. It is necessary, however, to point out in this place the uncertainty in which these researches have left the conception of endotoxins. They suggest that the toxic effects following upon the introduction of pathogenic bacteria into the animal body are not due to endotoxins, but are rather the result of the action of toxic cleavage products formed in the reaction between blood plasma and bacterial cell. These split products are not conceived as specific for individual bacteria but may be formed from all bacterial proteins, both the pathogenic and the non-pathogenic. The differences in pathogenicity between bacteria of this class would then depend entirely upon their powers to invade—not at all upon their possession of individually peculiar cell poisons. The differences in clinical course and toxemic manifestations would be taken to depend entirely upon the accumulation and the distribution of the invading germs, and the consequently variable energy in

[36] For a complete discussion of Vaughan's work see Vaughan, "Protein Split Products," Lea & Febiger, Phila. and N. Y., 1913.

the production of the toxic split products from them. Considerable experimental evidence has accumulated in favor of this point of view. But the subject is an involved one and will be considered more extensively in a later chapter.

The entire question of "endotoxins," or rather the problem of the mechanism by which such bacteria as typhoid bacilli, plague bacilli (and other organisms which do not produce exotoxins) poison the animal body, must be subjected to experimental revision. In addition to the idea of toxic split products in the sense of Vaughan and Friedberger, there are other alternatives. Jobling and Petersen have shown that bacteria injected into the circulation may absorb lipoidal substances which ordinarily act as anti-enzymes. In consequence of this, serum protease may be liberated to act upon the plasma itself, and produce toxic substances.

Again, it is well known that the bacterial cells are relatively poor in coagulable protein, and we have shown with one of our students (Aronovitch) that primary and secondary proteoses may be obtained in considerable quantities in bacterial extracts. It is not impossible that these in themselves may have toxic functions when liberated, without further splitting. This particular subject finds a more extensive discussion in the chapter on bacterial anaphylaxis.

Considerable difficulty in the explanation of bacterial intoxication has been encountered in infections caused by organisms like the streptococci, staphylococci, anthrax bacilli, etc., in which no true exotoxins and no toxicity of the cell bodies themselves have been demonstrable. The streptococcus question is perhaps the most interesting and illustrative of these problems. Individuals infected with streptococci, if the infection is at all severe, often show powerful toxic symptoms, and then lymphangitis which extends upward from the infected part in permanent red lines, is very likely a sign of toxic irritation rather than of ascending bacteria growth, inasmuch as a ligature applied across the part very rapidly causes a fading of the distal red line. Many authorities, Marmorek, Braun, Aranson and, more recently, Clark and Fenton and Havens have described true exotoxins for hemolytic streptococci to explain these phenomena. There is no doubt about the fact, as these studies as well as more recent studies by the writer and Miss Kuttner, and studies made in our laboratory by John Rice have shown that certain strains of hemolytic streptococci do produce a toxic substance which, in our own work, is designated as the "X" substance. This substance, however, is never quantitatively as powerful as many of the exotoxins and cannot, for the present at least, be classified with the true exotoxins. Incidentally, it can be washed off the surfaces of streptococci freshly grown on agar by rapid shaking with salt solution and filtration. Similar "X" substances can be washed away from fresh agar cultures of many other bacteria. Just how much toxicological

significance these substances have in the animal body, remains to be shown.[37]

A summary of bacterial toxemia would not be complete, we believe, unless we also mention a type of toxic reaction during bacterial infection which is dealt with more extensively in the chapter on bacterial anaphylaxis and the tuberculin reaction.[38] It appears from our own studies that in the course of many infections beginning within two weeks, the animal body becomes extremely hypersensitive to substances diffused out from the bacterial growth into the circulation. This is the basis of tuberculin, mallein, typhoidin, etc., reactions, and since these materials are actively produced by the growing bacteria, it is not at all unlikely that in infections sufficiently prolonged or repeated, much of the toxemia may be due to the action of these materials which are hardly toxic for the normal animal, but highly so for the animal sensitized by injection.

J. T. Parker,[39] working with the influenza bacilli, found that certain strains recently isolated after growing on chocolate broth for as little as 6 to 8 hours, will show in the broth filtrate a poison probably of this type, which may be so powerful that 1 to 2 c.c. will kill a rabbit acutely after 45 to 90 minutes. These poisons are thermolabile and while we do not believe that they belong to the exotoxin class, are, nevertheless, sufficiently powerful to perhaps explain many of the symptoms of the toxemia accompanying influenza bacillus infections.

In order to do injury to the infected individual the bacterial poisons must be produced in such locations that they can easily enter the physiological interior of the body. Few of the poisons that have been so far investigated can produce injury when introduced into the alimentary canal. In this location they are, as a rule, destroyed, or they pass through without doing harm. Neither diphtheria toxin nor tetanus toxin will produce symptoms when introduced intra-intestinally.[40] Even cholera poison does not pass through the uninjured intestinal wall. Kruse [41] assumes, and Kolle and Schürmann [42] seem to agree with him, that the absorption of cholera poison does not occur until the intestinal wall has been injured by the actual growth of the living bacteria. Kruse calls attention to experiments by Bürgers in which enormous quantities of

[37] For references on these poisons and a discussion of their significance, see Zinsser. *Jour. of Immunol.*, Vol. 5, p. 265, 1920.

[38] Zinsser. *Jour. Exper. Med.*, 34, 1921, 495.

[39] Parker, J. T. *Jour. Immunol.*, 4, 1919, 331.

[40] Meyer and Gottlieb. "Exp. Pharmakol.," Urban & Schwartzenberg, Berlin, 1911. Ransom. *Deutsche med. Woch.*, No. 8, 1898. Nencki. *Centralbl. f. Dakt.*, Vol. 23, 1898. Carrière. *Ann. de l'Inst. Past.*, Vol. 13, 1899.

[41] Kruse. "Allgemeine Mikrobiologie," Vogel, Leipzig, 1910, p. 934.

[42] Kolle and Schürmann in "Kolle u. Wassermann Handbuch," 2d Ed., Vol. 4.

cholera poison, i.e., 200 cultures of dead or living cholera bacilli, could be administered to healthy guinea pigs and rabbits by mouth without harm in spite of the fact that these animals are definitely susceptible to the poisons and although the poisons are not injured by the intestinal ferments. It is likely therefore that the absorption of poison begins only after the bactéria have extensively invaded the intestinal mucosa and, by injuring tissue, have opened paths for absorption. In the case of diphtheria probably a similar condition exists in that the localized injury to the mucous membrane at the point of lodgment of the primary infection prepares a portal of entry. The poison of the *Bacillus botulinus* seems to form an exception to this rule,[43] since this substance, though apparently a true bacterial toxin, is absorbed directly from the intestinal canal. With most bacteria this problem does not arise, since the poisons are elaborated within the tissues, where resorption is a necessary result.

Certain snake poisons, especially the viper poisons, may cause a violent gastro-intestinal irritation if taken by mouth, and the vegetable toxins, such as ricin, are characteristically of powerful toxicity if ingested. The toxic injury of the intestinal tract produced in the course of such infections as bacillary dysentery, typhoid fever, and some others is probably not due entirely to the action of the toxins directly in the intestine, but also to injury occurring in the course of excretion through the intestinal mucous membrane. There has been some controversy as to the probable reason for the innocuousness of most of the bacterial poisons after ingestion. Probably the action of intestinal bacteria is of relatively little importance as compared with the destructive action of the intestinal enzymes, particularly trypsin. There may also be an antagonistic action on the part of some of the constituents of the bile.

Like alkaloids and other organic as well as inorganic drugs, the action of many bacterial poisons is largely selective. Most of these poisons may excite inflammatory reactions if concentrated in any part of the body, but, in addition to this, there is a specific distribution after introduction which indicates that the poison goes into selective relationship with certain tissues and cells. This fact is most clearly illustrated by the bacterial hemotoxins which specifically injure the red blood cells of the infected individual and by such substances as the leukocidin produced by the *Staphylococcus aureus*, a poison which directly and visibly injures the white blood cells. Here the action is specifically aimed at a well-defined variety of body cell.

In considering this problem in connection with infectious disease, it is of great importance to distinguish between selective injury by the poisons transported through the body by the lymph, blood, and other channels, on the one hand, and the selective lodgment of the micro-organisms themselves on the other. The latter may occasionally de-

[43] Madsen in "Kraus u. Levaditi, etc.," Vol. 1.

pend on local cultural advantages for the particular bacteria in one organ or another, but may just as often be determined by the peculiar manner of entrance to the body which is most suitable for lodgment of the germs in question, and the degree of local resistance at the point of entrance, which determines whether or not the infection shall be locally limited or permitted to invade beyond this point. In the case of a disease like acute anterior poliomyelitis, where our knowledge of the micro-organisms which cause the disease is yet in its infancy, it is impossible to decide whether the injuries noted in the motor areas of the cord and medulla are due to toxins or the lodgment of the germs themselves. In the case of rabies it seems reasonably sure that the micro-organisms themselves select the nervous system. In such instances as the injury of the motor areas by tetanus poison, that of certain peripheral nerves by diphtheria toxin, or even the characteristic lesions of post-syphilitic maladies like tabes, we can be reasonably sure that we are dealing with the specific action of the poisons, independent of actual localized growth of the infectious agents.

Diphtheria toxin, after distribution through the body, may act upon many different tissues, as is evident by degenerations in the heart muscle, liver, and kidney, and the petechial hemorrhages in serous surfaces. In addition to this general action, however, there is a very marked selection of certain nerve centers. By Meyer and Gottlieb [44] diphtheria toxin is classed as a specific vascular poison. Its action results in a rapid sinking of the blood pressure with final cardiac death in spite of artificial respiration. These manifestations seem to have a central origin, with particular action upon the vagi and the phrenic nerves. Apparently also the localization of the diphtheritic lesion may influence the selection of individual nerves, the most concentrated action taking place upon the nerves whose endings are distributed in this particular region, for, as Meyer and Ransom [45] have shown, this poison, like tetanus toxin, may be absorbed into the nerves directly through the nerve endings. An interesting selective action also of diphtheria poison is the apparently specific alteration of the suprarenal glands which is regularly noticed, as enlargement and congestion, in diphtheria-infected guinea pigs, and which has been associated by many workers with the characteristic drop in blood pressure which accompanies all severe cases of the disease. Abramow [46] has studied this lesion particularly, and believes that it consists in a degeneration and final disappearance of

[44] Meyer and Gottlieb. "Pharmacology Trans. Halsey," Lippincott, 1914, p. 556.

[45] Meyer and Ransom. *Arch. de pharmacodyn.*, Vol. 15, 1905, also Meyer, *Berl. klin. Woch.*, 25 and 26, 1909, also *Arch. f. exp. Path. u. Ther.*, Vol. 60, 1909.

[46] Abramow. *Zeitschr. f. Imm.*, Vol. 15, 1912.

the chromaffin substance and of the medullary cells. He believes that this, together with degeneration of the heart muscle itself, is of great importance in causing the characteristic vascular failure.

In botulinus poisoning there is, as Marinesco [47] and Kempner and Pollack [48] have shown, a direct effect upon the cells of the anterior horns with degenerative changes in the Nissl granules.

Tetanus poison, which has been studied extensively by pharmacologists, shows a very marked affinity for the nervous system, as, in fact, the symptoms of tetanus indicate. Indeed, while many of the bacterial poisons are distributed by the blood stream to the point of final attack, in tetanus the absorption of the toxin from the lesion or the point of injection takes place entirely by the path of the nerves, entering by way of the motor nerve endings.

That this method of poison distribution might be, among others, an important one was suggested as early as 1892 by Bruschettini,[49] who found tetanus toxin in the nerves but not in the adjacent muscle and other tissues surrounding the point of subcutaneous injection. Similar results were obtained subsequently by Hans Meyer, whose experiments were confirmed and extended by Marie and Morax.[50] Finally Meyer and Ransom [51] furnished complete proof that the poison was absorbed from the blood and tissues by the peripheral nerve endings alone and was transported centripetally only by the paths of the neurons. The experimental facts elicited may be summarized as follows:

1. When tetanus toxin is injected into the thigh muscles of a guinea pig the poison is found at first only in the sciatic nerve of the same side and in the blood. (The determination of poison was made by injecting macerations of the respective tissues into mice.) If examination was delayed until the symptoms had become generalized, the poison was found in the opposite sciatic, but the muscle bundles, fat, etc., from the vicinity of the injection area were poison-free.[52]

2. When a nerve is cut poison absorption ceases as soon as axis cylinder degeneration has set in.

3. If the nerve is cut before the poison is injected the distal end contains poison, the proximal end does not. This again shows that the nerve absorbs the toxin not from its capillaries but solely through the end organs.

4. If a nerve which already contains poison is severed, toxin

[47] Marinesco. *Compt. rend. de la soc. de biol.*, Vol. 3, 1896.

[48] Kempner and Pollack. *Deutsche med. Woch.*, 32, 1897.

[49] Bruschettini. *Riforma medica*, 1892.

[50] Marie and Morax. *Ann. de l'Inst. Past.*, 1902.

[51] Meyer and Ransom. *Archiv f. exp. Path. u. Pharm.*, 49, 1903.

[52] In view of our discussion of the importance of fats in the absorption of tetanus toxin, it seems inconsistent that the toxin does not concentrate in fatty as well as in nervous tissues. This Meyer explains by the inactive and poorly vascularized condition of the fat tissues.

will disappear rapidly from the proximal end, since it no longer obtains a renewed supply from the periphery.

5. If antitoxin is injected into the nerve, above the point of injection, it will successfully bar the way for the ascending toxin.

6. Severing of the spinal cord prevents the passage of the poison from below upward.

These facts ascertained in the case of tetanus find their parallel in the phenomena of the distribution of rabic virus [53] as well as in that of poliomyelitis, in both of which there seems to be a progressive centripetal transportation through the nerves.

However, in these conditions we are probably dealing not with a poison but with a living virus and, though analogous, the conditions are not entirely comparable.

From the practical point of view these facts regarding tetanus may explain the frequent failure of therapeutic success attending the injection of tetanus antitoxin after the symptoms of the disease have set in, since in such cases the poison is already distributed to the nerves and is largely inaccessible to the antitoxin. They also have pointed a way toward a more hopeful therapy, namely, the method of injecting the antiserum directly into the nerves about the point of injury. It is not surprising, however, in view of the stated facts, that even this is unsuccessful when done at too late a time, after a considerable amount of poison has already passed above the point of injection to the spinal centers.

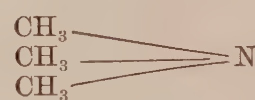
Such selective action on the part of the bacterial poisons is entirely analogous to the similar specific action of alkaloids, narcotics, and other drugs. In order that the poison may act upon a cell we must, of course, assume that it has either chemical or physical affinity for this cell. The problem, as many writers have pointed out, is strongly analogous to that of tissue staining. A dye must be able to form a chemical union with the cell or it must be soluble in the cell substance in order to stain it. The chemical difference between cells is a delicate one and not often definable by our present methods. We can obtain an insight into the principles probably underlying selective action only by inference from the relation between the chemical constitution of drugs or their physical properties, solubility, etc., and their respective tissue affinities. These problems are difficult and, to a large extent, obscure. They cannot be directly investigated upon bacterial poisons since these are themselves of chemically unknown nature. But the study of drugs of known constitution has revealed certain definite relations of this kind which have furnished analogies from which the general principles of selection in bacterial poisons can be surmised.

It is a well-known fact to pharmacologists that there is a definite

[53] Di Vestea and Zagari. *Fortschr. d. Med.*, Vol. 6, 1888.

relation between chemical structure and toxicity. Fraenkel [54] expresses it as follows: "By the addition of identical atom groups in an identical manner, similarly acting substances are obtained." He cites the well-known example of curare; whichever the path by which this poison is injected it leaves intact the tissues with which it comes in contact, but after general distribution acts specifically upon the nerve endings. It had been discovered by Brown and Fraser [55] that by introducing methyl radicles (CH_3) into molecules of various alkaloids, strychnin, morphin, atropin, and others, substances were obtained which paralyzed nerve endings, and this irrespective of their previous physiological action. It appears that the combination of four methyl radicles attached to the nitrogen atom (quaternary bases) universally possesses this paralyzing action. Tertiary bases on the other hand lack this property.

"Ammonium base"



"Tertiary base"

Quaternary

Subsequently Böhm [56] discovered that curare contains two bases --the one, "curin," is slightly toxic and is a tertiary base; the other, which possesses the typical curare action, "curarin," is an "ammonium base." By "methylizing" curin, curarin could be obtained.

From these and other examples it is clear that in a certain number of cases actual chemical affinity must play a part in toxic action; on the other hand, there are many cases in which toxic action seems to depend merely upon physical conditions such as solubilities. Meyer and Overton's well-known theory of narcosis maintains that certain narcotics exert their action by passing out of blood and lymph solution into solution by the fat-like, lipoidal substances (lecithin, cholestrin, etc.) contained in the nerve cells, because the latter are better solvents for them than is the blood plasma. This theory of Meyer and Overton has stimulated much investigation and speculation, and it is not unlikely that it is valid in the case of many narcotics, although it does not explain the action of narcotics in general; for Dickson notes that chloral hydrate, for instance, is more

[54] Sigmund Fraenkel. "Arzneimittel Synthese," 2d Ed., Springer, Berlin, 1906.

[55] Brown and Fraser. *Trans. Royal Soc. of Edinburgh,* 25, 1868, cited from Fraenkel.

[56] Böhm. *Arch. de Pharm.,* cited from Fraenkel. See also Dickson, "A Manual of Pharmacology," E. Arnold, London, 1912.

soluble in water than in oils, and some narcotic drugs like alcohol exert definite action on proteins and are oxidized in the body. These are pharmacological questions of which we cannot speak with authority. We wish merely to point out that the action of poisons upon the body may depend in some cases upon mere physical or mechanical relationship between the two.[57]

As regards bacterial poisons the union between poison and susceptible cell is extremely firm and difficult to dissociate in many instances, and this points to the possibility that, in these cases at least, true chemical union takes place rather than merely a loose combination like that of the solution of one substance in another. Furthermore, the complete inactivation of some poisons by mixture with the cells of tissues capable of binding them would likewise point to more than mere physical union. Nevertheless, it does not by any means exclude the thought that the poisons may, in fact, go into selective relationship with special cells because of physical properties, such as solubility in the lipoidal cell membranes,[58] and may subsequently be bound chemically or destroyed by oxidation or enzymotic hydrolysis after such entrance. In such a case the actual specificity would yet depend on purely physical properties.

In addition to the specific physical and chemical affinities between the poisons by certain cells there are probably also certain fortuitous factors connected with the distribution and local accumulation of the poisons which have some weight in determining the location of injury. For the specific selection is not absolutely strict and there are probably few parenchyma cells in the body that are entirely insusceptible to injury if the poisons are sufficiently concentrated upon them. Thus, to cite an analogy from the toxicology of non-bacterial poisons, in lead poisoning, as Meyer and Gottlieb point out, the paralysis of the extensors of the arm occurs chiefly in adults who use these muscles in the exercise of their professions (painters, type-setters), while in children and in animals, in which no such selective use of particular muscle groups is habitual, lead paralyses are atypical, attacking legs as well as arms. It is not unlikely that the frequent injury of the heart muscle by bacterial poisons or the irregular parenchymatous changes in various organs is determined by analogous fortuitous factors, in that functional activity and increased metabolism may predispose to injury.

Bacterial poisons also may produce their lesions in the course of excretion. This seems likely in the case of typhoid poisons in which

[57] Ivar Bang. "Biochemie der Lipoide," Bergmann, Wiesbaden, 1911. Meyer and Gottlieb. "Experimentelle Pharmakologie," 2d Ed., Urban & Schwartzenberg, Berlin, 1911.

[58] For Overton's theory of osmosis see R. Höber, "Physikalische Chemie der Zelle u. Gewebe," Leipzig, Engelmann, 1911. Compare also, regarding this entire question, the discussion in P. Th. Müller, "Vorlesungen über Immunität, etc.," Fischer, Jena, 1910.

we have often seen bloody diarrhea in rabbits within a few hours after intravenous injection of powerfully toxic culture filtrates. In connection with the dysentery bacillus Flexner and Sweet [59] have studied the conditions carefully. They succeeded in showing first that the introduction of the dysentery poison into the lumen of the intestine does no harm and that the toxin is slowly destroyed by peptic and tryptic digestion. They concluded that probably no absorption of the poison through the uninjured intestinal mucosa takes place. They then showed that the toxin after intravenous administration is excreted by the intestine and that the inflammatory reactions and injury of the mucosa are incident to this act of elimination.

Whether or not the kidneys are injured in the same way it is difficult to decide. In many infectious diseases, of course, the bacteria themselves pass through the kidney into the urine, and renal injury may result from the actual presence of the bacteria in the kidney; however, renal injury may also occur without this, and it is not at all impossible that the conditions here are similar to those just described for the intestine.

All the facts which we have considered indicate that, although most bacterial poisons can injure many different tissues, yet in some cases there is a particular susceptibility on the part of an individual tissue which is independent of accidental factors and seems to be due to specific chemical or physical affinity. It seems even that in tetanus, botulismus, and a few other conditions there is a differential selection of particular areas within a tissue like the nervous system, just as this occurs in the case of certain drugs. As stated above we have no satisfactory scientific explanation for this, but a great deal of work has been done to show that the bacterial poisons actually unite with and are taken up by the susceptible tissues.

Indirectly, proof of this has been brought by the demonstration of the rapid disappearance of various toxins from the blood streams of susceptible animals and their persistence in the circulation of animals insusceptible to them. Thus Dönitz [60] has shown that tetanus toxin injected into the blood stream of a susceptible animal rapidly diminishes in quantity, and Knorr,[61] in similar experiments, showed that the demonstrable disappearance of such toxins out of the blood stream is synchronous with the appearance of symptoms, a fact which excludes disappearance by excretion. Conversely Asakawa [62] showed that in pigeons, which are but slightly susceptible, tetanus poison could be demonstrated in blood, liver, spleen, kidneys, and muscles six days after injection, but not in the brain, showing

[59] Flexner and Sweet. *Jour. of Exp. Med.*, Vol. 8, 1906.

[60] Dönitz. *Deutsche med. Woch.*, No. 27, 1897.

[61] Knorr. *Fortschr. der Medizin*, 1897, No. 17, and *Münch. med. Woch.*, 1898, Nos. 11 and 12.

[62] Asakawa. *Centralbl. f. Bakt.*, Vol. 24, pp. 166 and 234.

that in this organ, at least, there must have been either a union or a destruction of the poison. Similar to these results are those of Metchnikoff,[63] who found the poison unchanged after two months in the circulation of insusceptible animals (lizards).

Direct evidence of union between susceptible tissues and poison has been furnished by the experiments of Wassermann and Takaki,[64] who showed that the brain and cord tissues of rabbits and guinea pigs, mixed with tetanus toxin before injection, served to neutralize its harmful effects. And it appears that the toxin-neutralizing property of the brain substances of various animals is proportionate to their individual susceptibility to the poison. Thus Metchnikoff [65] not only confirmed the results of Wassermann and Takaki for rabbits and guinea pigs, but showed further that the brains of chickens, animals that are but moderately susceptible, possess a correspondingly slighter neutralizing power, and, further, that brain tissues of entirely insusceptible cold-blooded animals, turtles and frogs, possess absolutely no neutralizing properties.

The original interpretation by Wassermann of these facts was based on the assumption that the poison was bound to the brain tissue just as it is bound to antitoxin. Experiments by Besredka [66] have cast some doubt upon this. This worker's experiments seem to indicate that a brain emulsion which has been saturated with the toxin can be rendered capable of absorbing more toxin if tetanus antitoxin is mixed with it. In other words, the affinity of the antitoxin for the toxin is stronger than that of the brain substance for the poison, and that the union toxin-brain tissue is very easily dissociated; as indeed it should if the union were purely a physical one depending on solubility.

After it had been shown that the poisons which acted specifically upon certain cells were actually taken up by these cells, a number of attempts were made to determine chemically the tissue element which united with the poisons. Noguchi [67] showed that cholesterin and alcoholic extracts of blood serum neutralized tetanolysin. The same thing was later shown by Müller,[68] and Landsteiner [69] showed that ether extracts of red blood cells likewise neutralized this poison. In a later study by Landsteiner and von Eisler [70] the relation of the tissue lipoids to various toxic substances was still more definitely established. They studied first the various hemolysins and found that extraction of blood cells with ether rendered the stromata less

[63] Metchnikoff. "L'Immunité dans les maladies Infect.," Paris.
[64] Wassermann and Takaki. *Berl. klin. Woch.*, 1898, No. 1.
[65] Metchnikoff. *Ann. de l'Inst. Past.*, 1898, p. 81.
[66] Besredka. *Ann. Past.*, 1903, p. 138.
[67] Noguchi. *Univ. Pa. Med. Bull.*, Nov., 1902.
[68] Müller. *Centralbl. f. Bakt.*, Vol. 34, 1903.
[69] Landsteiner. *Wien. kl. Rundschau,* 13, 1905.
[70] Landsteiner and von Eisler. *Centralbl. f. Bakt.*, 39, p. 318, 1905.

capable of binding the hemolytic substances. The same thing they showed for bacteriolysins, in the latter case demonstrating at the same time that the ether extracts of bacterial bodies possessed slight binding properties for the bactericidal substances of the serum. These experiments have, of course, a merely indirect significance in the present connection, since they do not deal with the type of poisons we have discussed. However, Landsteiner and Botteri [70a] also worked with tetanus toxin, and found that protagon obtained by alcoholic extraction of human brain possessed a powerfully neutralizing effect upon the toxin, markedly greater than that possessed by other lipoidal substances. 0.15 gram neutralized 120 minimal lethal doses of the toxin.

Takaki,[71] who investigated these relations in great detail, isolated an alcohol-soluble element, cerebron, from nerve tissues, a substance to which he ascribes the toxin-binding properties. Overton and Bang [72] found, furthermore, that cholesterin and lecithin inhibit the action of cobra venom, a poison which is in so many ways similar to those produced by bacteria. Taking into consideration all available evidence, we are forced to admit that the lipoids seem to play an important rôle in determining the selective action of the nervous system by the bacterial poisons. It may not, of course, be an influence depending merely upon the solubility of the harmful substances in the lipoids themselves. For, as Bang expresses it, "the lipoids possess to a high degree the property of altering by their presence the solubilities of other bodies," and it is quite possible that in the tissues they are present as lipoid-protein combinations. Their action in determining the solubility of toxins in a given cell may therefore be a purely indirect one.

It is of some interest in this connection to recall the experiments of De Waele,[73] which bring out another clear analogy between alkaloids and bacterial poisons in their relation to lecithin. He found that the addition of small quantities of lecithin increases the activity of both toxins and alkaloids in the animal body, whereas larger amounts inhibit both.

From the foregoing, then, it is plain that in order to gain entrance to the body and establish themselves there, certain criteria as to the pathogenic nature of the species of bacteria involved, and the virulence of the infecting strain as balanced against the susceptibility of the host, must coincide. According to the biological attributes of the bacteria, the path of entrance must be by a certain route adapted to their pathogenic habits. The infection thus having come about, further progress takes place, varying again according to

[70a] Landsteiner and Botteri. *Centralbl. f. Bakt.*, 42, 562, 1906.
[71] Takaki. *Beitr. zur chem. Phys. u. Path.*, 11, No. 19, 1908.
[72] See Ivar Bang, "Biochemie der Lipoide," Bergmann, Wiesbaden, 1911.
[73] De Waele. *Zeitschr. f. Immunit.*, Vol. 3, 1909, p. 504.

the biological properties of the bacteria; in some cases consisting very largely of local growth, tissue infiltration, extension and eventually, perhaps, entrance into the blood stream, distribution through the body, and in certain varieties of infections, secondary localization in the organs, with or without abscess formation. In other cases the organisms may remain localized, injuring the body by the production of a powerful poison. Between the two extremes, both factors may be active. Injury to the body, on the one hand, is by virtue of the local struggle which takes place between invaders and the tissue cells and fluids of the host, with injury to function and afterwards tissue destruction; and, on the other hand, by the absorption of toxic products of various kinds incident to the growth of the bacteria. These toxic products may be either the powerful and specific exotoxins, they may be products absorbed after liberation from the destroyed bacterial cells, or they may even be toxic substances produced by proteolytic cleavage of bacterial and tissue elements. The cause of death varies considerably. In the case of the true exotoxin formers, like diphtheria and tetanus, death will be due to the severe intoxication and the injury of specific cellular elements necessary to life, the local, mechanical causes being negligible. However, even in diphtheria, when the localization of the process is such that it obstructs the respiratory passages, especially the larynx, a purely mechanical death may be possible. This, however, is a special case.

The cause of death in the cases of bacteria in which the toxin formation is of a less severe degree is not absolutely clear. There may be extensive tissue degenerations in organs like the liver, the kidney, the heart muscle, etc., which eventually lead to death. There may be, as in some cases, as very severe malaria, perhaps anthrax and other infections in which the parasitic organisms become enormously increased in number, an actual interference with capillary circulation. In many cases, such as meningococcus infection, there may be actual mechanical and local toxic injury to parts of the central nervous system which lead to death.

The causes of death by bacterial infection, therefore, may be manifold, but it is not likely that even in cases like streptococcus, anthrax and staphylococcus infections where we have but very rudimentary knowledge of poison formation, that the toxic element is ever entirely absent.

CHAPTER III

OUR KNOWLEDGE CONCERNING NATURAL IMMUNITY, ACQUIRED IMMUNITY, AND ARTIFICIAL IMMUNIZATION

NATURAL RESISTANCE AGAINST INFECTION

In the preceding chapters we have confined ourselves largely to the consideration of those properties of the bacteria which determine their ability to infect. In this discussion, however, we have repeatedly emphasized the fact that every infectious disease is the result of a struggle between two variable factors—the pathogenic powers of the bacteria on the one hand, and the resistance of the subject on the other, each of these again modified by variations in the conditions under which the struggle takes place. Thus a given micro-organism may be capable of causing fatal infection in one individual but may be only moderately virulent or even entirely innocuous for another. Conversely the same individual may be highly susceptible to one variety of bacteria, but highly resistant to others. Even in reactions with one and the same micro-organisms, the susceptibility or resistance of the individual may be determined by variations in the physiological state or by the environmental conditions under which the two factors—invader and invaded—are brought together. Therefore, the conceptions "resistance," "immunity," and its opposite "susceptibility," are relative terms which can never be properly discussed without careful consideration of all modifying conditions which influence them.

The science of immunity deals with a detailed analysis of these variables. Its ultimate practical aim is the determination of methods by which an original susceptibility can be transformed into resistance or even immunity. And the rational method of approaching this subject consists in a careful study of the conditions of susceptibility and immunity as they exist naturally in the animal kingdom.

The mere fact that both animals and man are in constant contact with infectious micro-organisms, many of them in a high state of virulence, indicates in itself that the animal disposes normally over a defensive mechanism of considerable efficiency.

To a certain extent, of course, this escape from harm is due to the external defences of skin and mucous membrane which, in the

healthy state, mechanically prevent the entrance of the micro-organisms into the body. For we have seen, in another place, that few of the bacteria can pass through the uninjured surfaces. Moreover, added to this, there is some protection in the bactericidal properties of the secretions. An example of this is the inhibitory power exercised by the acidity of the normal gastric juice upon the cholera spirillum. In order to infect the intestinal canal of guinea pigs with these organisms Koch found it necessary to neutralize the gastric juice with sodium carbonate solutions, and other observers have found it necessary to inject directly into the duodenum. But even after entrance into the animal tissues a second line of defence is normally encountered by all invading germs which tend to inhibit their further progress more or less perfectly. This active opposition to the bacteria after their entrance is expressed chiefly in the antibacterial (bactericidal) activity of the blood serum, and the phagocytic powers of leukocytes and other cells. To a certain extent these forces are active against all bacteria in all animals, but they may vary in different species, races, or even individuals in potency against any given infectious agent, and, to a certain extent, variations in resistance may be referable to this. The analysis of these forces, both in the normal and in the artificially immunized animal, forms the substance of the systematic discussions which are to follow, and, for the present, we will confine ourselves to an examination of the facts that have been gathered regarding the actual differences in normal resistance or "Natural Immunity" between various species of animals.

And if we glance over the list of diseases to which different species and races of animals are victim, it is immediately evident that some animals are never spontaneously infected with many of the micro-organisms that cause extensive and fatal ravages in others. Also, within the same race or species, an epidemic sweeping through a community will kill many individuals and leave others unscathed. Such differences point to variations in the defensive mechanism, since the invader in these cases is the same. We speak, therefore, of *Natural Immunity* which is an attribute of *species,* that which, within the same species, is *racial,* and that which, within the same race, is *individual.* And the attempts to discover the causes underlying such differences in natural resistance have elucidated many of the fundamental principles of immunity in general.

Instances of natural immunity which appear to depend on species are common. We have pointed out, above, that in order to make infection at all possible, it is necessary that the invading germ shall find suitable cultural conditions in the body of the host. It is this simple principle which probably explains the fact that bacteria which cause disease in warm-blooded animals cannot, as a rule, cause disease in those that are cold-blooded, and *vice versa.* Thus frequent

attempts to produce anthrax in turtles, frogs, and other cold-blooded species have failed. Also among warm-blooded animals differences in body temperature have been shown to influence susceptibility. Thus avian tuberculosis does not develop in mammals, nor do the human and bovine types of tubercle bacilli infect birds. And this is probably due to the fact that the avian bacillus has become adapted to growth at from 40° to 45° C., about the normal temperature of birds, while the mammalian bacilli cease to grow when the temperature is raised above 40° C. Another observation which clearly illustrates the influence of body temperature upon susceptibility is that made by Gibier [1] upon anthrax. Frogs are ordinarily resistant to this disease. When they are kept in water at 35° C. a fatal infection can be produced. Nuttall's [2] experiments with plague infection in lizards illustrate the same point. Kept at 16° C., no infection could take place. Warmed to 26° C., they could be readily infected. It is ordinarily assumed that these results are explicable upon the basis of purely cultural and temperature considerations. And this, indeed, is most likely. It is possible, however, that an additional factor involved in this may be the lowering of the general resistance of cold-blooded animals when warmed, just as warm-blooded animals can be rendered susceptible by chilling.

It is for similar simple cultural reasons, possibly, that diseases which occur spontaneously in carnivora do not occur in purely herbivorous animals. The relative resistance of dogs to anthrax and to tuberculosis may possibly be accounted for in this way. However, there are many micro-organisms which infect easily both carnivorous and herbivorous animals, and it may well be that the frequently cited cases we have mentioned above depend on factors more complicated than mere cultural conditions incident to metabolic differences. In most cases of species resistance, indeed, simple nutritional conditions alone do not serve as valid explanations.

Species resistance may be so perfect that it amounts to an absolute immunity. This is apparently so in the cases cited above, namely the immunity of the cold-blooded species to certain diseases of warm-blooded animals. However, such examples are exceptional. When we are dealing with diseases of warm-blooded animals only, natural resistance, in all but a limited number of cases, is sufficient only to prevent the spontaneous occurrence of the particular disease, or to prevent infection when experimental inoculation with moderate doses is practiced upon normal animals. In most of these cases, however, when the dose experimentally administered is excessive, or the resistance is lowered artificially, by chilling or any other form of local or general injury, infection can be accomplished. In the case of protozoan diseases species adaptation is much more rigid

[1] Gibier. *Compt. rend. de l'acad. des sc.*, Vol. 94, 1882.
[2] Nuttall. *Centralbl. f. Bakt.*, Vol. 22, 1897

and parasites that infect one species are very often restricted entirely to that class, being unable to infect any other animal, even though no striking difference in temperature or metabolism exists.

We may convey the clearest conception of all such species differences by a tabulation of some of the more important infectious diseases of man with a statement in each case concerning its transmissibility to animals, as follows:

Tuberculosis, human type, spontaneously infects man. It is very often observed in monkeys kept in captivity. Cattle, swine, and sheep are probably never spontaneously infected; guinea pigs are highly susceptible to experimental inoculation. Cattle, swine, sheep, and rabbits are relatively very resistant to experimental infection. Dogs and goats are still more so. Birds seem to be entirely refractory.

Tuberculosis, bovine type.—Spontaneous infection occurs in domestic animals, chiefly cattle; it is less frequent in sheep, hogs, and horses; it has been reported in dogs and goats. In man infection *does* occur, but only a small percentage of human tuberculosis is of the bovine type, and these cases are almost exclusively in children. In tabulating 1,042 cases which have been carefully studied, Park and Krumwiede [3] report the following figures:

Cases of Tuberculosis in Man (1042)
- Over 16 years
  - Human type 677, bovine type 9.
- 5 years to 16 years
  - Human type 99, bovine type 33.
- Under 5 years
  - Human type 161, bovine type 59.

The large majority of bovine infections were abdominal or involved cervical lymph nodes.

Experimental infection is successful in rabbits and guinea pigs, both of these animals succumbing more rapidly to this than to the human bacillus. In fact, the relative resistance of rabbits to the human bacillus is such that rabbit inoculation is one of the most important methods of differentiating between the two types. Birds are refractory.

Tuberculosis of the avian type occurs spontaneously in birds. It may be experimentally produced in rabbits (Strauss and Gamaleia). Injected into cattle it causes a local reaction only.

Tuberculosis of cold-blooded animals is not transferable to warm-blooded animals.

Syphilis spontaneously occurs in man only. It can be inoculated into chimpanzees, in which primary and secondary lesions develop, corresponding mildly to human syphilis. Primary lesions can be

[3] Park and Krumwiede. *Jour. of Med. Res.,* Vol. 23, 1910.

produced in lower monkeys. It can be transferred by intratesticular inoculations to rabbits.

Gonococcus infection occurs spontaneously in man only. No typical lesions can be produced in experimentally inoculated animals, though death can be caused by large doses, probably by toxic action.

Influenza bacillus spontaneously infects man only. Experimental infection is partly successful in monkeys only. (Pfeiffer and Beck, *Deut. med. Woch.*, 1893.)

Glanders.—Spontaneous infection occurs in horses and mules; less frequently in sheep, goats, and camels. This disease, like plague, may be regarded as primarily a disease of animals, but man may be infected by direct or indirect contact with the diseased animal. All domestic animals may be infected experimentally with ease, except cattle and rats, in which cases large doses are necessary. Birds show local reactions only. (Wladimiroff—in "Kolle und Wassermann Handbuch," Vol. 5, 2d Ed.)

Plague occurs spontaneously chiefly in man and in rats. It has also been found in California ground squirrels and in hogs during plague epidemics in Hong Kong. It is highly infectious for guinea pigs and white rats—slightly less so for mice; rabbits are much less susceptible than guinea pigs. Dogs, cats, and cattle are relatively resistant. Birds appear to be immune. Cold-blooded animals are immune unless artificially warmed. (See above.)

Malta fever occurs spontaneously in man and in goats. It is pathogenic for all mammals, but it is not fatal for lower animals when the organisms are directly cultivated out of the human body.

Diphtheria occurs spontaneously in man only. Experimental inoculation is fatal in guinea pigs, rabbits, dogs, cats, and birds. Rats and mice are highly resistant. The typical pseudomembranous inflammation can be produced in susceptible animals only after previous injury of the mucous membrane, and then it rarely shows any tendency to spread.

Tetanus is spontaneous in man, horses, cattle, and sheep. It is found rarely in dogs and goats. Birds are highly resistant to experimental inoculation.

Anthrax is primarily a spontaneous infection of cattle, sheep, and horses; it occurs in man largely through direct or indirect contact with these animals. Guinea pigs, rabbits, and white mice are very susceptible to experimental inoculation. Rats and hogs are less susceptible, and dogs are relatively resistant, though they can be regularly killed by moderate doses intravenously injected. Birds and cold-blooded animals are highly resistant.

Asiatic cholera develops spontaneously in man only. Rabbits and guinea pigs can be killed by injections of cultures, but die probably of toxemia. In rabbits a cholera-like condition has been pro-

duced by injection of the spirilla into the duodenum after ligation of the common bile duct. (Nikati and Rietsch, Ref. in *Deut. med. Woch.,* Vol. II, 1884, p. 613.) Ordinarily no multiplication takes place in the animal body. Pigeons are insusceptible, a fact which helps to distinguish this organism from *Spirillum metchnikovi* and other similar bird-pathogenic spirilla.

Typhoid fever occurs spontaneously in man only. It has recently been produced in a mild form in chimpanzees. Animals are susceptible to the endotoxins and can therefore be killed by injections of bacilli and extracts, but the organism is not invasive as in the case of the lower animals. Typhoid septicemia can be produced in rabbits by inoculating them with especially virulent cultures of the bacilli, or cultures previously grown on rabbit-blood agar (Gay). The typhoid-carrier state may ensue for considerable periods in such animals.

Pneumococcus infection in various forms occurs spontaneously in man. Rabbits, mice, and guinea pigs are highly susceptible. Rats, dogs, cats, cattle, and sheep are relatively resistant.

Straphylococcus and streptococcus infections may occur in almost all of the warm-blooded animals, chiefly as abscess producers. In horses a severe form of pleuropneumonia is caused by them.

Leprosy occurs spontaneously in man only. Lesions simulating human leprosy have been produced in monkeys by inoculation, and partially successful experiments have been made upon the Japanese dancing mouse. Other animals are immune.

Scarlet fever occurs spontaneously in man only. Monkeys may possibly be susceptible, though not all observers have been successful in such experiments. (Draper and Handford, *Journ. of Exp. Med.,* Vol. 17, 1913.) Landsteiner and Levaditi (*Ann. Past.,* Vol. 25, 1911) have succeeded in producing the disease in the chimpanzee, though they failed with lower monkeys.

Small-pox occurs spontaneously in man only. It is probably identical with cow-pox. (See reasons for this assumption given by Hacius as cited by Paul in "Kraus and Levaditi Handbuch," etc., Vol. 1.) It can be experimentally produced in monkeys.

Measles develops spontaneously only in man. *Macacus rhesus* has been successfully inoculated by Anderson and Goldberger (U. S. Pub. Health Reports, 26, 1911). Other animals are immune.

Typhus fever occurs in man only. Experimentally it has been produced in chimpanzees, *Macacus, Cercopithecus, Ateles,* and *Mycetes* monkeys. Anderson has succeeded in producing temperature reactions in guinea pigs by injecting blood from typhus patients or from other similarly infected guinea pigs. More exact information concerning this disease will probably be available soon, if the reported cultivation of the organism of the disease by Plotz is authenticated.

Yellow fever up to the present has been observed in man only.

Poliomyelitis is spontaneous in man only. Can be transmitted to monkeys and—in a doubtful form—to rabbits. No other animals are known to be susceptible.

The above represents an incomplete tabulation of the variations in susceptibility in the animal kingdom for infections which occur spontaneously in man. They will illustrate sufficiently, however, the facts of variable species susceptibility as we have stated them. We might, with equal profit, tabulate the infections occurring spontaneously in any single species of animal and show how variable would be their pathogenic powers for other animals and for man. Thus man is immune to the organism which causes cattle plague, and to that of chicken cholera, and probably to many other diseases peculiar to animals, though, of course, in the case of infections of the human being we are entirely dependent for such information upon observed immunity to spontaneous infection, and upon a few instances of accidental inoculation.

In regard, also, to differences of susceptibility between various *races,* within the same species, many interesting facts have been observed. Thus gray mice are, as a rule, more resistant to streptococcus and pneumococcus infection than are white mice. Algerian sheep are said to be more resistant to anthrax than are European sheep. Of black rats inoculated by Müller [4] with anthrax over 79 per cent. survived, while of white rats similarly inoculated only 14 per cent. survived.

In man, too, racial differences are marked. The extraordinary susceptibility of the negro to tuberculosis is familiar to all American physicians, and it is well known that Eskimos transported to temperate climates and civilized conditions are particularly prone to contract this disease. Small-pox is considered a relatively mild disease in Mexico. Dr. James Carroll [5] stated that whites are more susceptible to yellow fever than are negroes, and that among the latter those living nearest the equator are less susceptible than are the more northern races. There seems to be no doubt about the actual occurrence of such racial differences, although, as Hahn [6] very justly points out, many instances formerly regarded as racial differences of susceptibility may have been simulated by racial, or often religious, differences of custom that influence sanitary conditions, and consequently the incidence of epidemic disease.

Apart from the explanations furnished in a few instances by gross physiological differences such as body temperature, the factors determining species resistance are largely a mystery, and in the

[4] Müller. *Fortschr. der Med.,* 1893. Cited from Sobernheim, in "Kolle u. Wassermann Handbuch," 2d Ed., Vol. 3.

[5] Carroll in "Mense, Tropenkrankheiten," Vol. 2, p. 124.

[6] Hahn in "Kolle und Wassermann's Handbuch," Vol. 1.

matter of racial variations, of course, we have no instances in which such very obvious physiological factors play a part. In attempting to find causes for differences of resistance or susceptibility in general, the nature of the problem makes it necessary for us to examine it from a number of different points of view. A micro-organism may be infectious for a given species of animal more than for another, because of special adaptation to the conditions, nutritive and otherwise, encountered in the tissues of these animals. Such adaptation is illustrated in the experience of Pasteur with "rouget" and with rabies, where passage through one variety of animal enhanced the virulence for this species but reduced it for others; and the same thing is easily demonstrated in the laboratory with so many bacteria that it may be accepted as a principle underlying enhancements of virulence in general. This adaptation implies that, to a certain extent, the part played by the animal body in determining its own susceptibility is passive. Gonococcus, for instance, infectious for man only, requires human protein for growth, at least in its first generations outside the body. Its ability to cause disease in man may be largely dependent upon its cultural need of human protein. The resistance of other animals to this disease, then, is, in part, due to their failure to supply proper nutriment. This, as Kolle points out, is analogous to *Atrepsie,* a term used by Ehrlich, in speaking of the insusceptibility of one species to cancerous growths originating in another.

Again, "adaptation" on the part of the bacteria may imply, not only an increased ability to meet altered cultural conditions, but an actual acquisition of greater offensive or invasive powers with which to meet the particular defences opposed to it by the given animal. Thus the increased virulence of typhoid bacilli after cultivation in immune sera would point toward an increased ability to survive under the adverse conditions encountered in the animal body. An organism may possibly acquire particular infectiousness for one species to the exclusion of others, by a succession of spontaneous inoculations—comparable to the experimental passage of the micro-organism through animals of the same species. This is especially probable in diseases such as gonorrhea, syphilis, and some others where infection is usually direct from one person to another. And it is these diseases particularly in which infectiousness is rather strictly limited to the human species.

Inheritance of Immunity.—Regarding the matter purely from the point of view of the animal body and the factors which determine its powers to ward off a given infection, we may justly assume that natural resistance may be largely a matter of inheritance. Whether this is to be interpreted as purely an instance of survival of the fittest or whether immunity acquired by an individual can be wholly or in part transmitted to the offspring is an open question—at pres-

ent in the same state of unclearness as are other questions relating to the transmissibility of acquired characteristics. However this may be, there are a number of facts available which indicate that inheritance plays an important part. It is apparent in the case of many diseases afflicting human beings that infection takes a milder course in those races among which it has long been endemic —whereas the same disease, suddenly introduced among a new people, is relatively more severe and spreads more rapidly. This seems to be the case with yellow fever and tuberculosis, and in measles and small-pox, too, the principle seems to hold good. Syphilis when first described authentically—as epidemically sweeping through Europe toward the close of the 15th century—appears to have been a far more acute and violent disease than it is among us to-day. It may well be that this depends upon a gradual elimination (elimination in this case, especially as far as reproduction is concerned) of those individuals that are fortuitously more susceptible and, by natural selection, a higher racial resistance is gradually developed. Whether or not direct inheritance of the individually acquired immunity can be considered at all as a contributing factor is difficult to decide. That immunity can be transmitted from mother to offspring was observed by Chauveau [7] as early as 1888. Lambs thrown by anthrax-immune ewes possessed a higher resistance against this infection than did the lambs of normal ewes. The extensive experiments of Ehrlich,[8] carried out chiefly upon mice with the vegetable poisons ricin and abrin, showed that in these cases immunity may be transmitted from mother to offspring, but depends upon a passive transfer of the specific antitoxins both by the blood and the milk of the mother. The sperm of the father did not seem to have anything to do with inherited resistance, since no immunity followed in the offspring when immunized males were paired with normal females. From the complete absence of immunity in the second generation (grandchildren) of the immunized female, and from the short duration (2 to 3 months) of its persistence, he concluded that the ovum itself had no influence, but that the entire phenomenon was attributable to a passive transference of antitoxins from mother to child during gestation and lactation. He interpreted, in the same sense, Chauveau's anthrax experiments, and similar experiments of Thomas [9] and Kitasato [10] with symptomatic anthrax, suggesting that, here also, a transfer of antibodies from mother to offspring had taken place. The experiments of Ehrlich permit of no doubt as to the validity of his conclusions. However, we must remember that they were carried out with antitoxic immunity only, in which the

[7] Chauveau. *Ann. Pasteur,* 1888.

[8] Ehrlich. *Zeitschr. f. Hyg.,* 1892, Vol. 12.

[9] Thomas. *Compt. rend. de l'acad. des sc.,* Vol. 94, cited by Ehrlich, *loc. cit.*

[10] Kitasato. Cited by Ehrlich, *loc. cit.*

resistance is purely dependent upon the circulating antibody and is never, even in actively immunized individuals, a permanent state.

The ricin work of Ehrlich, then, constitutes nothing more than an example of the transfer of antibodies from mother to offspring, a passive immunization which in no sense can be regarded as a true somatic inheritance of immunity. This question of the transference of antibodies from mother to offspring has given rise to a considerable amount of investigation, a most extensive piece of work on the transmission of hemolytic antibodies emanating from the laboratory of Famulener,[11] who came to the conclusion that in goats, at least, such antibodies are chiefly transmitted with the milk, and not through the placenta. The question is of considerable importance in connection with the transfer of diphtheria antitoxin from mother to child in human beings, where we shall see in a later chapter that an amount of antitoxin sufficient to protect is often found in the new-born child, and lasts in adequate amount up to, or nearly up to the first year of life. Since Famulener's work, most pediatricians have assumed that such transfer in human beings took place through the milk, more especially through the colostrum during the first days of feeding, and much weight was given to this opinion by the observations of Theobald Smith [12] concerning the importance of colostrum in preventing a premature colon bacillus infection of the bowel in calves. Apparently calves that never have had colostrum, but were brought up from the beginning without nursing from the mother, in many cases succumb to a disease called scours, consisting of a generalized colon bacillus infection. A recent investigation in our own laboratory by Drs. Kuttner and Ratner [13] has shown that in human beings the conditions are unlike those existing in the calf. Careful measurement of antitoxin values in colostrum and milk of mothers and in the cord blood have shown that whatever antitoxin is transmitted, passes through the placenta, hardly any being found in the colostrum. To our minds, in spite of much that has been written to the contrary of recent years, these experiments, carefully carried out, should settle the question of maternal transmission to the child through the placenta, at least in human beings. It is quite likely that the conditions in human beings may differ from those prevailing in many animal species, because of the anatomical differences between the human placenta and that of many lower animals. In the human being there is only one layer of connective tissue between the maternal of the fetal blood in the placental layers; whereas, in animals, two, three and even four cell layers may intervene.[14]

[11] Famulener. *Jour. Inf. Dis.*, X, 332, 1912.
[12] Theobald Smith. *Jour. Exp. Med.*, August, 1922.
[13] Kuttner and Ratner, in press.
[14] Grosser. "Eihaüte u. Plazenta," Braumüller, Leipzig, 1909.

In immunity such as that acquired against typhoid fever, plague, cholera, and other diseases after recovery from an attack, the individual remains relatively resistant long after the demonstrable antibodies have disappeared from the circulation, and we must assume that this permanent resistance depends upon a physiological alteration—inexplicable for the present, but surely residing in the body cells. In such cases it is by no means certain that there may not be a very slight, but through generations gradually acumulating, inheritance of immunity. At any rate the experiments of Ehrlich do not disprove such a possibility. Moreover, in this connection it must not be forgotten that natural immunity, unlike acquired immunity, cannot be passively transferred from one animal to another, and implies therefore a fundamental cellular difference rather than a condition depending merely upon antibodies circulating in the blood.

For this last reason also it has been unsatisfactory to attempt explanations of natural immunity purely upon grounds of bactericidal and other properties of the blood serum. These points we will take up at greater length when we discuss the mechanism of resistance in general.

An important observation upon the inheritance of serum properties is that which has been made by Ottenberg and Epstein [15] in connection with the iso-agglutinins. We shall see in another section that the blood serum of one human being will often possess the property of agglutinating the human blood cells of another individual. These iso-agglutinating constituents of the serum are apparently transmitted from parents to offspring. Von Dungern and Hirschfeld,[16] in studying these iso-agglutinins in 72 families, upon 348 people, not only confirmed the observations of the preceding workers, but showed that such inheritance follows Mendelian laws. Not only is this of great biological interest, but it is of great importance in connection with our present discussion in showing that such properties as agglutinating powers of serum can be influenced by inheritance from the father as well as from the mother.

Individual Differences of Resistance in Same Race.—The individual differences in resistance which unquestionably exist among members of the same species and races are very difficult to explain, but, as far as we can tell anything about them at all, they seem to depend upon variation in what is popularly spoken of as "general condition." The laboratory animals with which most experimentation is done, rabbits and guinea pigs, if healthy, show very slight individual variations. In fact, the astonishing uniformity of reaction on the part of guinea pigs of similar age and weight against measured quantities of bacterial toxins has alone made it possible

[15] Ottenberg and Epstein. *Proceedings of the N. Y. Path. Soc.*, 1908.
[16] Von Dungern and Hirschfeld. *Zeitschr. f. Immunitäts.*, Vol. 4, 1910.

to utilize these animals in the standardization of antitoxins. Pneumococcus and streptococcus cultures can be measured with reasonable accuracy upon white mice of approximately uniform weight, and the same animals are relatively uniform in their reactions to identical amounts of tetanus poison. Many other examples might be cited which make it clear that healthy animals of the same species, kept under the same conditions, fed upon the same food, and of approximately the same age and weight, differ but slightly from each other in reaction to the same infectious agent. This would indicate that the individual differences in resistance displayed so plainly by human beings are due, not to any *fundamental* individual variations, but rather to such fortuitous factors as nutrition, metabolic fluctuations, temporary physical depression, fatigue, or chilling. A person suffering from functional impairment of any kind is more likely to permit the invasion of a pathogenic micro-organism than is a perfectly healthy well-nourished individual of the same species.

Most of these facts we know from the accumulated experience of clinicians who also have given us much valuable information concerning the susceptibility to infection on the part of chronically diseased persons, especially diabetics and nephritics. In the case of a few of these influences, chilling and fatigue, experimental data on animals are available. It is, however, extremely difficult to analyze the causes underlying such depression of resistance. For instance, with fatigue or chilling there may be temporary congestion of mucous surfaces, due to vasomotor influences, which alter the secretions on mucous surfaces, or interfere with the normal mobilization of leukocytes, permitting penetration of bacteria where ordinarily they would have been held back. Our ignorance is nowhere more clearly illustrated than in the fact that we know practically nothing concerning the relation between a thorough chilling and the acquisition of what is spoken of as a common "cold." We can only assume that there is interference in some way with the normal bactericidal and phagocytic mechanisms, making possible the penetration and lodgment of small quantities of bacteria, ordinarily destroyed immediately after entrance or prevented from entering at all.

Of course we must except those individual differences of susceptibility which may be dependent upon inheritance. We know, for instance, that in such diseases as diphtheria, where resistance depends upon antitoxins circulating in the blood, there may be a passive immunity, conferred from mother to offspring, which lasts for several weeks or months after birth. It is important to remember such a possibility in the selection of guinea pigs for diphtheria antitoxin standardization, as Anderson has pointed out. Whether or not a tendency to tuberculosis can be inherited is still an open question. In most cases it is more than probable that the supposedly inherited tendency to tuberculosis is not really an inherited sus-

ceptibility, but rather an actual infection acquired during childhood from the parents. Cornet and Kossel,[17] who have recently summarized the statistics dealing with this problem, have come to the conclusion that this factor, namely, infection from the parents, probably is the cause of the greater frequency of tuberculosis among children of tuberculous parents, and that there is no definite proof of inherited susceptibility.

ACQUIRED IMMUNITY AND IMMUNIZATION

We have outlined in the preceding pages the differences in susceptibility to various diseases apparent among different species of animals, and have noted that the degree of resistance of some animals to infection with germs rapidly fatal to others is often sufficiently well-marked to be termed "immunity." Such immunity, because it is a natural biological attribute of the species, as much a characteristic property as are its anatomical or physiological properties, has been spoken of as *"Natural Immunity."*

It is a matter of common knowledge, however, that among species of animals readily susceptible to certain infections resistance, or even extreme resistance, i. e., immunity, may be acquired by an attack of the disease. Thus human beings who have recovered from plague, small-pox, typhoid fever, cholera, the exanthemata, mumps, typhus, yellow fever, and a number of other conditions do not ordinarily contract the disease a second time. In some of these conditions, notably cholera, plague, typhoid fever, and small-pox, the rule is almost invariable. In others, such as measles, scarlet fever, and mumps, a second attack may occur, though it is rare. A few infections like pneumonia and influenza may recur at relatively short intervals.

The following table briefly indicates infectious diseases in which permanent immunity follows an attack:

Infectious Diseases in Which One Attack Conveys Lasting Immunity

Plague.
Typhoid—second attack rare—about 2.4 per cent. (Curschmann).
Cholera.
Small-pox—second attack very rare.
Chicken-pox—second attack very rare.
Scarlet fever—second attack about 0.7 per cent.
Measles—second attack uncommon, but less rare than scarlatina.
Yellow fever.
Typhus fever.
Syphilis—reinfection rare, though more common than formerly supposed.
Mumps—second attack rare (Kraus).
Poliomyelitis.

[17] Cornet and Kossel in "Kolle u. Wassermann," Vol. 5, 2d Ed.

No lasting immunity is conferred by one attack in:

Infection with the Pyogenic cocci
- Gonorrhea
- Pneumonia
- Influenza
- Glanders
- Dengue fever
- Diphtheria in general protection, second attack in 0.9 per cent. cases—0.01 antitoxin unit per c. c. of circulating blood protects
- Recurrent fever
- Tetanus
- Erysipelas
- Beri beri
- Tuberculosis

There is another group of diseases in which the immunological conditions after infection are as yet not clear—namely, protozoan infections like malaria and trypanosomiasis, and treponema diseases like syphilis. In these conditions reinfection seems to be impossible only so long as the individual still harbors the micro-organism, but no lasting immunity is conferred. We have discussed these conditions in extenso in the section on syphilis. In tuberculosis, also, resistance to superinfection is dependent on the presence of a focus.

These observations actually form the point of departure of that entire branch of medical science which devotes itself to the study of resistance to infection, serum diagnosis, and specific therapy, and it will be seen that all the facts that have been gathered upon these subjects are the fruits of detailed analysis of this phenomenon of acquired immunity.

Its occurrence in many instances has been so striking that ancient observers, long before the birth of rational medicine, referred to it, and often drew from it conclusions of great hygienic importance. Thucydides, in the second book of his account of the Peloponnesian Wars, in describing the plague at Athens, notes the apparent safety from reinfection of those who had recovered, suggesting the possibility of their being therefrom immune against disease in general. The literature of the Middle Ages and of earlier modern times contains numerous further references which indicate that acquired resistance was clinically recognized as a result of recovery from many diseases. The phenomenon was not only observed, but put to practical utilization by the ancients of China and India. Thus the practice of inoculating children with small-pox material from the active pustules of patients, or making them sleep in beds or wear the shirts of sufferers was a dangerous practice but logical, on the reasoning that the disease conveyed to a person in full health and good condition would probably take a mild course and confer

immunity, while the naturally acquired disease, contracted often because of the weak and debilitated condition of the individual, would be more apt to end fatally.

Such methods, though barbaric and eventually unjustified by the naturally high mortality incident upon them, were actually brought to Europe from the East, and for a time practiced in European countries.

The first great advance which bridged the gap between the observations regarding naturally acquired immunity and rational experimental imunization was made by Edward Jenner. It had been noticed before Jenner began his work that milkmaids and others who had contracted cow-pox in the course of their occupations were usually spared when a small-pox epidemic occurred in their community. Sporadic attempts had been made to put this observation to practical use, but no one with sufficient intelligence, persistence, and training had taken up the matter seriously. Jenner, interested by the reports of this nature and by his own observations, was especially impressed by the similarity between the local manifestations of small-pox, cow-pox, and a disease of horses spoken of as "grease." Though at first disinclined to identify small-pox with cow-pox (at present the prevailing opinion is that the second is an attenuated form of the former), Jenner thoroughly investigated cases of alleged protection by cow-pox, a claim which before this had been hardly more than a rumor, and finally, with the encouragement of John Hunter, proceeded to the vaccination of human beings with cow-pox, testing the result by subsequent inoculation of the same individual with small-pox. His report to the Royal Society in 1796 and his subsequent publications incorporate the results of these experiments by means of which the practice of vaccination against small-pox was introduced and the virtual eradication of the disease from civilized communities was attained.

The principles underlying small-pox vaccination are extremely simple. The attenuated virus after inoculation incites a mild and localized form of the disease, from which the subject recovers rapidly and completely. The recovery implies the mobilization of certain protective forces and a specific physiological alteration of the body in such a way that a permanently, or at least prolongedly, increased resistance against the disease remains. In consequence, if the individual is subsequently exposed to spontaneous infection with this disease, his acquired specific resistance suffices to prevent invasion by the virus. This is merely an artificial imitation of the conditions which obtain when an individual recovers from an attack of a disease and is rendered immune thereby. In this case, however, the attenuation of the virus has eliminated the dangers attendant upon an actual attack. The immunity thus conferred is probably never as perfect nor as lasting as that following a seizure of the disease in its unattenuated

form; however, it suffices, as a rule, to prevent spontaneous infection which is never as severe a test as experimental inoculation.

In contrast to the "Natural Immunity" which is an inherited attribute of race or species, we speak of such increased resistance in a member of an originally susceptible race as "Acquired Immunity." When the immunity has been attained as the result of an attack of the disease itself it is spoken of as *"Naturally or Spontaneously Acquired Immunity."* When produced by some form of treatment with the virus of the disease, altered in such a way that an actual attack is averted, we speak of it as *"Artificially Acquired Immunity."*

The premises of Jenner's reasoning were valid as his experiments were convincing. But knowledge regarding infectious disease and its causation by living germs was not developed until almost one hundred years later, by the work chiefly of Pasteur. For this reason no direct continuation of Jenner's work appeared until Pasteur made his communication upon Chicken Cholera to the Parisian Academy of Medicine in 1880. Though his investigations differed entirely from those of Jenner both in method and the nature of the disease with which they dealt, Pasteur recognized the similarity of the fundamental principles underlying both discoveries.

His observations took origin in a purely accidental occurrence. Cultures of chicken cholera which had been allowed to stand without transplantation and under aerobic conditions for periods of several months were found to have diminished in virulence. Inoculated into chickens, they failed to kill, giving rise in many cases to localized lesions only. It occurred to Pasteur that inoculation with such an attenuated culture might protect against subsequent infection with fully virulent strains and, indeed, experimental investigation of this idea proved to be correct. He developed a method of "vaccination" against chicken cholera which consisted in injecting first a very much attenuated culture of the organism (*premier vaccin*), and, after 12 or 14 days, another less perfectly attenuated (*deuxième vaccin*), since he observed that a single inoculation was often insufficient to confer protection. After two inoculations a degree of immunity could be attained which sufficed to protect against spontaneous infection as well as against experimental inoculation with doses of the virulent germs, fatal for untreated animals.

These experiments, simple as they are, constitute the beginnings of the science of Immunity, since, for the first time, an investigator working with a pure culture of a pathogenic micro-organism had succeeded, in planned and purposeful experiments, in conferring artificial immunity. The path was now clearly indicated and the years immediately following were fruitful in the development of many methods by which pathogenic bacteria may be attenuated and changed in such a way that they can be used to confer immunity without causing more than a transient and harmless reaction in the

subject. Most of the earlier discoveries of this kind came from Pasteur himself and from members of his school.

Since in all these methods the inoculated animal attains its increased resistance by reason of the activities of its own tissues, these processes are spoken of as "*Active Immunization.*" No protective factor is conferred directly. The disease itself is inoculated, though in an altered form, and the subsequent immunity is purely the result of the physiological reaction occurring as the subject struggles against and overcomes the injected virus, bacteria, or their products. Such "Active Immunization," we shall see, is in contrast to "*Passive Immunization,*" a procedure in which the serum of an actively immunized animal is injected into another, carrying with it certain substances by which protection is conferred. The recipient here is passively protected by products of the active reaction which has taken place in the body of the donor.

After his success in active immunization against chicken cholera Pasteur applied the principles here learned to experiments upon the protection of animals against anthrax. This problem was fraught with considerable difficulty because of the great virulence of the anthrax bacillus. However, successful attenuation was attained by a method which depended upon the cultivation of anthrax cultures at temperatures above the optimum for its growth. Toussaint [18] had shown that the resistance of sheep could be increased if they were inoculated with blood from animals dead of anthrax after this had been heated to 55° C. for ten minutes. Toussaint's idea had been that by heating the blood in this way the bacteria themselves were killed. Pasteur [19] showed, however, that this was not the case, but that what actually occurred was a reduction of the virulence of the strain by the exposure to heat. As a matter of fact, morever, the method of Toussaint did not furnish a reliable means of attenuating anthrax, and Pasteur succeeded in developing a far more satisfactory procedure on which he based a practical method for the protective vaccination of sheep and cattle.

His method was as follows: [20] Virulent anthrax bacilli were cultivated at 42° to 43° C. on neutral chicken bouillon (Sobernheim states that horse or beef broth—or even agar—answers the same purpose). Cultivated under these conditions a gradual and progressive reduction of virulence occurs. After about 12 days of such cultivation the culture as a rule no longer kills rabbits, but is still virulent for guinea pigs and mice. After twenty-four or more days the virulence for rabbits and guinea pigs is lost and mice only can be

[18] Toussaint. *Compt. rend. de l'acad. des sc.*, 1880.

[19] Pasteur, Chamberland and Roux. *Compt. rend. de l'acad. des sc.*, Vol. 91, 1881.

[20] Cited from Sobernheim. "Kraus und Levaditi Handbuch der Technik, etc.," Vol. 1, 1909.

killed with it. The latter—the most fully attenuated strain—was called *premier vaccin* by Pasteur, and, in the immunization of cattle or sheep, is first injected. After 10 or 12 days the stronger *deuxième vaccin* is administered. This is the method which Pasteur used in his now classical experiments at Pouilly-le-Fort, in which he convinced a hostile audience of the efficacy of his immunization. Sheep were protected in the manner indicated, and 14 days after the last injection a fully virulent culture was inoculated and the animals found capable of successfully resisting it.

In the train of this work many other methods of producing active immunity have been devised—all of them of considerable theoretical interest and many of them practically adapted to some special case. We may conveniently classify these methods as follows:

I. Immunization with Living but Attenuated Cultures

(1) Methods in which the attenuation is obtained by heating. This is the method of Toussaint as outlined above, in which anthrax blood was heated to 55° C. for 10 minutes, and is probably the least efficient or reliable method for the attenuation of the anthrax bacillus. It has been applied to rabies by Babes (cited from Kraus in "Kraus u. Levaditi Handbuch, etc.," Vol. 1, p. 708), who attenuated the virus by heating to 58° C. for periods varying from 2 to 40 minutes.

(2) Attenuation by prolonged cultivation of the bacteria at temperatures above the optimum for their growth. This is illustrated by Pasteur's anthrax immunization as described in the preceding paragraphs.

(3) Attenuation by passage through animals. Examples of this are Pasteur's experiments with the "rouget" organism, in which passage through rabbits diminished the virulence for hogs. The attenuation of rabic virus by passage through monkeys is another instance, and Jennerian vaccination is also an example of this, although here the attenuation by passage through cattle is attained naturally and not by experimental procedures. Based on the same principle is Behring's [21] method [22] of immunizing cattle against tuberculosis by inoculating them with tubercle bacilli of the human type.

(4) Attenuation by prolonged growth of bacteria on artificial media in the presence of their own metabolic products. This is the method first employed by Pasteur in chicken cholera, as described above, and is applicable to many organisms, such as pneumococci, streptococci, and others. In fact, it is difficult to maintain the virulence of many of these bacteria unless special methods of cultivation

[21] Behring. "Therapie der Gegenwart," April, 1907.

[22] See also Römer, "Kraus u. Levaditi Handbuch," 1st Suppl., p. 310.

or passage through animals are practiced. Pasteur believed that free access of oxygen to the cultures increases the rapidity of the attenuation.

(5) Attenuation by drying. The classical example for this method is the Pasteur method of prophylactic immunization against rabies. Rabbits are inoculated with *virus fixe,* and their spinal cords dried for varying periods in bottles containing KOH at a temperature of about 25° C. The virus grows progressively weaker with each day of drying. Greater details concerning this method are given in another place.

(6) Attenuation by the use of chemicals.—Chamberland and Roux [23] succeeded in attenuating anthrax by growing it in the presence of various antiseptics. They used carbolic acid 1 to 600, bichromate of potassium 1 to 1,500 and sulphuric acid 1 to 200, and found that, after a short time of cultivation under such conditions, the bacilli lost their ability to form spores and became avirulent for sheep. Behring [24] and others have applied this method to the attenuation of diphtheria toxin; Behring adds terchlorid of iodin, Roux potassium iodid—iodin solutions. The principle, of course, is not exactly the same in the last cases, since here the attenuation is not of the bacteria themselves, but rather of the toxin.

(7) Attenuation by cultivation under pressure. This method is difficult to apply, and has no striking advantages over other procedures. It was employed by Chauveau [25] for the attenuation of anthrax. He succeeded in accomplishing this by cultivation of anthrax bacilli at 28° to 39° C. at a pressure of 8 atmospheres.

II. Active Immunization with Fully Virulent Cultures in Sublethal Amounts

The original methods of Pasteur carried out with chicken cholera and anthrax were aimed particularly at diminution of virulence, since these organisms, as isolated from the diseased animal, are so extremely infectious that it would be very difficult—(in the case of many animals, impossible)—to inoculate with the unattenuated germs without producing fatal disease. However, in the case of many other infections it has been found feasible to inoculate normal animals with the fully virulent germs in such small quantities that the body can successfully overcome them, and, in doing so, acquire specific resistance. It is obvious that this method is more easily carried out with the organisms which Bail terms "half parasites" than with organisms as highly infectious as anthrax. Ferran [26]

[23] Chamberland and Roux. *Compt. rend de l'acad. des sc.,* 96, 1882.
[24] Behring and Wernicke. *Zeitschr. f. Hyg.,* 12, 1892.
[25] Chauveau. *Compt. rend. de l'acad. des sc.,* Vol. 98, 1884.
[26] Ferran. *Compt. rend. de l'acad. des sc.,* 1885.

applied this method both to animals and to human beings with broth cultures of cholera spirilla. Högyes [27] has introduced a similar procedure for immunization against rabies by injecting dilutions of fully virulent rabic virus, beginning with a dilution of 1 to 10,000 and rapidly working up to a dilution of 1 to 10. In tuberculosis immunization with fully virulent cultures in small amounts has been attempted by Webb, Williams, and Barber,[28] using the Barber method of isolation, and giving a single micro-organism at the first injection. That such a method is feasible, if carried out with sufficient care, even with the most virulent germs, was demonstrated by the same workers. They succeeded in immunizing animals against anthrax (with cultures kept 12 hours on agar) [29] by injecting a single thread (3 to 6 bacilli) as the first dose, and then gradually increasing the amount.

In the general laboratory immunization of animals treatment with virulent bacteria in sublethal doses is of considerable value and frequently employed.

It would seem that possibly this method or some modification of it will be found to have very definite advantages over methods in which either attenuated or dead bacteria are employed. Bail's work upon the aggressins and upon anti-aggressin immunity (see chapter I, page 17) has opened the possibility that virulent bacteria provide, within the living body, specific aggressive substances which are not produced in the test tube. If this proves to be true, and the question is by no means settled, it may be necessary in such cases to immunize with organisms which are in a condition capable of producing these aggressins. Sublethal doses of fully virulent organisms would furnish these conditions more perfectly than attenuated avirulent strains, in which the invasive (aggressive) power is considerably diminished.

The methods of active immunization so far described differ from those which are to follow in that the preceding were all based upon the use of living bacteria or virus, whereas the methods to be described below depend upon the treatment of animals with dead bacteria or bacterial products. It is well to call attention in this place to the fact that a number of recent investigations seem to point to the greater efficiency of immunization with living germs. This method has recently given hopeful results in the case of plague in the hands of Strong,[30] and Metchnikoff and Besredka,[31] in their attempt to vaccinate chimpanzees against typhoid fever, make the

[27] Högyes. "Lyssa Nothnagels Handbuch, etc.," Vienna, 1897.

[28] Webb, Williams, and Barber. *Jour. of Med. Res.*, Vol. 15, 1909.

[29] This was not possible where the organisms were taken directly from the blood of a dead mouse. In such cases even a single thread caused fatal disease.

[30] Strong. *Jour. of Med. Res.*, May, 1908.

[31] Metchnikoff and Besredka. *Ann. Past.*, Vol. 25, 1911.

statement that vaccination with dead typhoid bacilli or autolysates does not confer adequate protection, but that this can be attained by treatment with small doses of the living bacilli.

In speaking of this subject it is well to mention recent observations upon immunization with *"sensitized"* bacteria,[32] although this necessitates anticipatory reference to subjects not so far discussed. It is a matter of common experience in laboratories that rabbits and other animals will withstand relatively large amounts of pathogenic bacteria if these are first treated with heated specific immune serum (sensitized). This is probably due to the fact that such "sensitized" micro-organisms are very rapidly taken up by phagocytes. In spite of the phagocytosis, immunity is developed. Metchnikoff and Besredka, in the communication alluded to above, state that typhoid vaccination with unaltered living bacilli is efficient, but is attended by severe local and general reactions. If the living bacilli are first "sensitized" no such severe reaction occurs and immunization is nevertheless successful. The recent work of Gay points in the same direction, and it is at least possible that by the practice of sensitization we may be able to employ living unattenuated organisms for purposes of immunization more extensively than we have in the past.

III. Active Immunization with Dead Bacteria, and Bacterial Extracts

This method is the one most extensively practiced in the laboratory immunization of animals. It is usual in most experiments of this kind to inject dead organisms once or twice before living bacteria are administered. High degrees of resistance can in some instances be attained by progressively increasing doses of dead cultures only. This method is not only useful in experimental work, but is clinically employed in the active immunization of human beings as introduced by Wright and as applied, before Wright, to tuberculosis (tuberculin treatment). But it is very probable that the immunity so attained is not entirely comparable to the immunity following an attack of a disease, nor even that produced by the injection of living bacteria.

The method employed for killing the bacteria is of considerable importance since, both by excessive heating as well as by too vigorous chemical treatment, the immunizing properties of the bacterial protein may be destroyed. In employing heat it is a safe rule never to expose the bacteria for prolonged periods to temperatures which considerably exceed the thermal death point. As a rule, heating non-

[32] See discussion on "sensitization" of antigens in chapter VI.

spore-forming bacteria to a temperature of from 65° to 70° C. for thirty minutes will suffice to kill them without too radically altering the immunizing properties of the protein constituents.[33]

If the temperature is not raised above 60° C., and this is advised by many workers, the suspensions must be carefully controlled by cultural tests before they are used, at least for the treatment of human beings. As we shall see in a later section, the best results have been obtained when heating was not carried beyond 53° to 55° C.

When bacterial death is to be accomplished by chemicals the antiseptics most commonly used are carbolic acid (0.5 per cent.), toluol (removed before use of vaccine by filtration or evaporation), chloroform, and formaldehyd (1 per cent.).

Pfeiffer, who was one of the first to practice the immunization of animals with dead bacteria on an extensive scale, believed that, in the case of bacteria which were toxic by reason of their intracellular constituents (endotoxins), the injection of the cell protein itself, whether dead or alive, was the sole essential for successful immunization. The method developed by Kolle [34] and by Pfeiffer and Marx [35] for the prophylactic immunization of human beings against cholera depends upon the injection of cholera cultures emulsified in salt solution, killed by exposure to 58° C. for one hour, and further insured against contamination by the addition of 0.5 per cent. phenol. The application of this method to other diseases, both prophylactically and therapeutically, is more fully discussed in another place.

Since the essential point in such immunization is the introduction of the bacterial protein, it is often customary to inject bacterial extracts instead of the whole cells. This has been especially desirable in the case of such insoluble micro-organisms as the tubercle bacillus, where the injection of the whole dead organism produces localized reactions similar to those caused by the living bacteria.[36] Thus "Old Tuberculin," as commonly used, is a glycerin-broth extract of tubercle bacilli. The method has been extensively used and a variety of procedures have been devised for bacterial extraction. These have included simple autolysis of the bacterial bodies in alkaline broth, shaking in salt solution in mechanical shakers, trituration with salt or sand, trituration after freezing, digestion with proteolytic enzymes, and extraction by pressure in a Buchner press.

[33] In a subsequent chapter we shall see that the physical changes produced in an antigen by heat result in differences in the antibodies formed after animal inoculation. This point has practical significance in the present connection. See also the chapter on agglutinins, the work of Joos there discussed, and Friedberger and Moreschi, *Centralbl. f. Bakt.*, 1905, Vol. 39.

[34] Kolle. *Deutsche med. Woch.*, 1897, p. 4.

[35] Pfeiffer and Marx. *Deutsche med. Woch.*, 1898.

[36] Prudden and Hodenpyl. *N. Y. Med. Journal*, 1891.

We may mention some of the more important methods for preparing bacterial extracts for purposes of immunization and antigen production in general as follows:

A. Extraction of Bacteria by Permitting Them to Remain for Prolonged Periods in Fluid Media

The bacteria may be grown upon slightly alkaline bouillon and kept at incubator temperature for one to two months. They are then filtered through Berkefeldt or other suitable filters. This is the common method of producing antigen for precipitin reactions, in fact the method employed by Kraus in the discovery of the bacterial precipitins. It is by no means certain whether the antigens prepared in this way represent simple extractions or autolytic products of the bacteria; probably both processes take place. The antigenic value of the fluids obtained in this way is never very great. From such filtrates Brieger and Mayer, Pick, and others have attempted to obtain the antigen in a purified form by chemical precipitation. Pick [37] precipitates the bouillon filtrate by saturation with ammonium sulphate; the precipitate is redissolved in water and again precipitated with ammonium sulphate and the resultant precipitate dried on a filter. It is then dissolved in water and precipitated with alcohol. The sticky substance which comes down represents the antigen.

Suitable extracts can occasionally be obtained also by emulsifying agar cultures in physiological salt solution and allowing them to stand for twenty-four hours or more at incubator temperature. In our own experience we have found this method rather inefficient for yielding strong extracts. More efficient extraction is usually obtained when the bacteria are suspended in alkaline fluids such as $\frac{N}{10}$ sodium hydrate. Lustig and Galleotti digest the bacterial mass for 24 hours with 1 per cent. NaOH, then precipitate with ammonium sulphate, dry *in vacuo* and pulverize.[38]

Recently, also, Uhlenhuth [39] has employed the proprietary preparation "antiformin" [40] for the production of antigens. This

[37] Pick. "Hoffmeister's Beiträge, etc.," Vol. 1, 1902. For an extensive discussion of the various methods employed for the production of bacterial antigens by chemical methods see Pick in Kraus und Levaditi, etc., Vol. 1, and the same author in Kolle u. Wassermann, etc., 2nd Ed., Vol. 1.

[38] See Pick. *Loc. cit.*

[39] Uhlenhuth. *Centralbl. f. Bakt.*, I, Ref. Vol. 42, Beilage, p. 62.

[40] "Antiformin" is a substance largely employed for the cleansing of pipes and vats of organic matter because of its powerfully solvent action. Its value in concentrating tubercle bacilli out of sputum and other mixtures depends upon its power to dissolve the tissue elements and all bacteria except

substance thoroughly dissolves all but the acid-fast bacteria when used in concentrations of 2.5 per cent. Since it is alkaline it is necessary to neutralize it with hydrochloric or sulphuric acid before use.

For the preparation of antigen from pneumococci Neufeld [41] has utilized the solvent action upon these organisms of bile. He adds the bile and broth cultures just as this is done in the diagnostic "bile test" (0.1-0.2 c. c. of fresh bile to a broth culture; sodium taurocholate solution can also be used). Many bacteria can also be broken up by emulsifying them in 17 per cent. salt solution and allowing them to stand for some time in this medium and then diluting this to 0.85 per cent.

B. Extraction by Mechanical Methods

One of the most useful methods for obtaining extracts of bacteria within a relatively short time is that which Besredka [42] has applied mainly for the preparation of typhoid (endotoxin), 24-hour agar cultures washed up in very small quantities of physiological salt solution, killed by heat at 60° to 65° C. and dried *in vacuo*. The dried mass is mixed with a measured quantity of dry salt and the mixture thoroughly triturated in a mortar for a considerable time. While triturating distilled water is added in small quantities until the fluid represents a 0.85 per cent. salt solution. This is allowed to stand for anywhere from a few hours to a week, and the bacteria are then removed by centrifugalization. This method has been modified by many observers and gives good results whenever thorough trituration is practiced. It is also probable that the exposure to the hypertonic salt solution in the earlier stages of the trituration may aid considerably in breaking up the bacteria.

Trituration after freezing is a method which has yielded excellent results in the hands of Macfadyen and others. This requires a rather complicated piece of machinery originally described by Macfadyen and Rowland. The principle of this is one of mechanical trituration in a steel cylinder which is surrounded by an ice-brine mixture so that the bacteria and sand may be kept frozen during the process.

those that are acid-fast. Rosenau ("Preventive Medicine and Hygiene," Appleton, 1913, p. 1020) gives its composition as follows: "Antiformin consists of equal parts of liquor sodæ chlorinate of the British Pharmacopœia and a 15 per cent. solution of caustic soda. The formula for liquor sodæ chlorinate is as follows:

Sodium carbonate	600
Chlorinated lime	400
Distilled water	4,000"

[41] Neufeld. *Zeitschr. f. Hyg.*, Vol. 34, 1900.

[42] Besredka. *Ann. de l'Inst. Past.*, 19-20, 1905, 1906.

Mechanical trituration is also the principle of the production of the new tuberculins as advised by Koch.

One of the earliest methods of obtaining bacterial substances by mechanical means was that used by Buchner and Hahn [43] in the production of their "plasmines." The bacteria were grown in quantity on large agar surfaces, the moist bacterial masses triturated together with quartz and were then subjected to high pressure in an especially constructed press spoken of as the "Buchner press."

Mechanical breaking up and extraction of the bacteria also underlies in principle the use of the variously constructed shaking machines. There are many models of such machines on the market, all of them designed to accomplish prolonged agitation of bacterial emulsions. In many cases the apparatus can be placed inside of an incubator and shaking carried on at 37.5° C. The bacteria are suspended for this purpose in distilled water salt solution, weak alkali, or in serum, and glass beads or sand may be added to aid in their mechanical injury. Shaking must be continued for 24 hours or more in order to give good results.

IV. Active Immunization with Bacterial Products (Toxins)

As soon as the investigations of Roux and Yersin had shown that in some diseases, at least, the injury sustained by the infected animal was largely due to the soluble toxins produced by the bacteria, it was logical to attempt to immunize animals with such products. Probably the first attempts in this direction were those made by Salmon and Smith in hog cholera. The experiments of these writers have attained much historical importance since they represent the first purposeful attempt to immunize animals with the products of bacterial metabolism. In the actual experiment, however, the immunization practiced by Salmon and Smith was probably a combination of immunization by bacterial products and by dead bacteria. Nevertheless, the thought of immunization with bacterial products was the underlying one in their experiments. Working with the hog cholera bacillus which they had recently discovered they immunized pigeons in the following way: The bacilli were grown in broth for two weeks, and the cultures were killed by exposure to 58° to 60° C. for several hours. One and one-fifth cubic centimeter of this culture liquid was then injected into pigeons, and after three such injections the inoculated pigeons withstood, without harm, doses of the bacilli which rapidly killed untreated animals. Salmon and Smith [44] stated distinctly in their conclusions that: "Immunity may

[43] Buchner and Hahn. *Münch. med. Woch.*, 1897.

[44] Salmon and Smith on "A New Method of Producing Immunity from Contagious Disease," *Proc. Biol. Soc.*, Wash., D. C., III, 1884, 6, p. 29, printed Feb. 22, 1886.

be produced by introducing into the animal body such chemical products (results of bacterial growth in culture fluids) that have been produced in the laboratory.[45]

Similar attempts to immunize rabbits against certain forms of septicemia by the injection of culture filtrates were made by Chamberland and Roux [46] in 1888,[47] and the same investigators applied this method to anthrax immunization just prior to the discovery of diphtheria toxin by Roux. However, neither in hog [48] cholera [49] nor in the other infections upon which this method was first tried do the bacteria produce a true soluble toxin, and the immunization which was accomplished depended probably upon the injection of bacterial extracts. Nevertheless, these attempts had shown the way in a new direction, and bore immediate fruit in the investigations of Brieger and Fraenkel,[50] and more especially in those of Behring [51] and his collaborators. Fraenkel, though following the method of injecting filtered diphtheria culture fluids, came to the erroneous conclusion that the toxin and the immunizing substances in the cultures were not identical (loc. cit., p. 1135).[52] The degree of immunity obtained in his experiments, moreover, was a slight one only.

Behring, in his first work, in collaboration with Kitasato, succeeded in immunizing animals with culture filtrates and with pleural exudates of diphtheritic animals. Similar results were accomplished with tetanus. Since the publication of these results—especially in consequence of the epoch-making discovery of passive immunization, to which they were the immediate guides, toxin immunization has been investigated and accomplished in all cases in which a true soluble toxin can be demonstrated. It has accordingly been carried out with the exotoxins of pyocyaneus [53] bacilli, the bacilli of symptomatic anthrax [54] and botulinus,[55] the specific leukocidins [56]

[45] For a copy of the original paper by Salmon and Smith I am indebted to Professor Theobald Smith.

[46] Chamberland and Roux. *Ann. Past.*, Vol. 1, 1888.

[47] *Op. cit.*, Vol. 2, 1889.

[48] Joest in "Kolle u. Wassermann Handbuch, etc.," Vol. 3, p. 632.

[49] Karlinski. *Zeitschr. f. Hyg.*, Vol. 28, 1898.

[50] Brieger and Fraenkel. *Berl. kl. Woch.*, 1890, Nos. 11, 12 and 49.

[51] Behring and Kitasato. *Deutsche med. Woch.*, No. 49, 1890. Behring. *Deutsche med. Woch.*, No. 50, 1890. Behring and Wernicke. *Zeitschr. f. Hyg.*, 1892.

[52] De Schweinitz, indeed, who further studied hog cholera immunization (*Medic. News*, 1892; *Centralbl. f. Bakt.*, Vol. 20, 1896), claimed that in sterilized milk the bacillus produced "enzymes" with which immunization could be accomplished.

[53] Wassermann. *Zeitschr. f. Hyg.*, Vol. 22.

[54] Kempner. *Zeitschr. f. Hyg.*, Vol. 23, 1897.

[55] Grassberger and Shattenfroh. *Deuticke*, Wien, 1904.

[56] Denys and Van der Velde. "La Cellule," Vol. 2, 1895.

produced by staphylococci, and with various bacterial hemolytic poisons (tetanolysin and other bacterial hemotoxins). The result of all this work has been the very important determination that susceptible animals may be actively immunized both against the effects of the toxin alone, as well as against the virulent bacteria themselves, by systematic treatment with culture filtrates containing the toxins. Since in many cases the effects of the toxins were so powerful that their attenuation was desirable, Behring and others have advised the addition of iodin terchlorid and other chemicals to the first injections.

PASSIVE IMMUNIZATION

In the logical development of the fundamental facts regarding immunization, with attention focused early on the blood and body fluids as the probable carriers of immunity, it was but a rational step from active immunization to the conception that such acquired immunity might be transferred from a treated to a normal animal by injecting blood from the former into the latter. This was probably the underlying thought of Toussaint's [57] early work with anthrax, in which he heated anthrax blood to 55° C. and injected it into other animals, wrongly believing that the bacteria had been killed by the heating. The method of Toussaint, however, was vague in its conception, and in no way constitutes an example of true passive immunization. The beginning was made in a purposeful and clearly conceived way by Richet and Héricourt.[58]

These investigators actively immunized dogs against staphylococci, and then attempted to transfer the immunity to normal rabbits by injecting defibrinated blood from the immune dogs. Their success was a partial one only, for reasons that we will discuss directly. Reasoning similar to that of Richet and Héricourt was applied by Babes and Lepp [59] to rabies immunization. When the blood of rabies-immune dogs was injected into normal dogs and rabbits, and these inoculated with rabies several days later, the treated animals regularly survived the controls, but in one dog only was the occurrence of rabies absolutely prevented. Since their animals were not experimentally inoculated, but subjected to the more uncertain method of allowing them to be bitten by a mad dog, and since the series included 4 animals only (2 treated and 2 controls), Babes and Lepp were unable to draw definite conclusions. The establishment of passive immunization as a proved scientific fact was finally

[57] Toussaint. *Compt. rend. de l'acad. des sc.*, 1880.

[58] Richet and Héricourt. *Compt. rend. de l'acad. des sc.*, 1888, Vol. 107, p. 750.

[59] Babes and Lepp. *Ann. Past.*, Vol. 3, 1889.

accomplished in 1890-1892 by Behring and Kitasato,[60] and by Behring and Wernicke. The results of this work—the direct outcome of their success in actively immunizing with soluble toxins, is summarized in their first paper as follows: "The blood of tetanus-immune rabbits possesses tetanus-poison-destroying properties; these properties are demonstrable in the extravascular blood and in the serum obtained from this; these properties are of so lasting a nature that they remain active in the bodies of other animals, so that one is enabled to obtain positive therapeutic results by transfusing the blood or injecting the serum. These tetanus-poison-destroying properties are absent from the blood of non-immune animals, and when the tetanus poison is inoculated into normal animals it can be demonstrated as such in the blood and other fluids of these animals after death."

With these researches begins the therapeutically practicable method of passive immunization which is now in such widespread and successful use in the treatment of diphtheria, in the prophylactic treatment of tetanus, and, to a less common and less successful degree, in the treatment of dysentery, typhoid fever (Besredka), plague, and a number of other bacterial diseases. The same method has been successful in the treatment of various diseases of domestic animals. The principle was also applied by Ehrlich [61] to ricin and crotin immunity, in the formulation of which he succeeded in working out passive immunization on a quantitative basis, showing that the degree of immunity in such cases could be directly referred to the amounts of the specific antitoxin present in the blood of the immunized animal. Calmette,[62] and Physalix and Bertrand,[63] then succeeded in producing passive immunization against snake venoms.

To summarize the success of passive immunization in general we may say that it has achieved its greatest usefulness in the case of those diseases in which the pathogenesis depends upon a true exotoxin—which, as we have mentioned before, leads to the formation of an antitoxin in the immunized animal. In these cases the passive immunization is accomplished by the transfer of the antitoxins from the treated to the normal animal.

In the case of bacterial infections in which no true toxin is formed—where no antitoxin results and the immunity depends, as we shall see, upon an enhancement of the bactericidal and phagocytic properties of the blood and the cells, passive immunization has not been a practical therapeutic success. The probable reasons for this cannot be properly discussed until we have examined more closely into the mechanism by which the immune animal is protected after

[60] Behring and Kitasato. *Deutsche med. Woch.*, No. 49, 1890.
[61] Ehrlich. *Deutsche med. Woch.*, 1891; *Fortschr. d. Med.*, p. 41, 1897.
[62] Calmette. *Compt. rend. de la soc. de biol.*, 1894.
[63] Physalix and Bertrand. *Compt. rend. de la soc. de biol.*, 1894.

specific treatment with bacteria or their products. The moderately beneficial effects of the various antiplague sera and the limited success attending the use of antistaphylococcus, antistreptococcus, and antipneumococcus sera probably depend, as recent work tends to show, not upon the direct action of antitoxin bodies, but rather upon the indirect opsonic action [64] which renders the bacteria more easily amenable to phagocytic action. These points we shall discuss at greater length in a succeeding chapter.

The Conception of Specificity. In speaking of methods of immunization in the preceding sections we have frequently employed the terms "specific" and "specificity," without sufficiently defining them. It will be necessary to explain them since the principle of specificity is at the same time one of the most important and one of the most mysterious of the phenomena of immunity. When an individual has recovered from an attack of typhoid he is thereafter immune to typhoid—but to no other disease—similarly with plague, cholera, small-pox, etc. The same principle governs artificial immunization. Vaccination against anthrax protects against anthrax only—and active or passive immunization in any of the infectious diseases produces a resistance which is "specifically" aimed only at the particular infectious agent with which the original active immunity was produced. The principle points to an exquisite chemical difference between the protein substances which constitute the bacterial cell bodies or their metabolic products. For although by chemical methods we can detect no differences between them—yet the reactions of immunity are sharply differentiating. When we come to consider the antibodies which specifically precipitate the substances by which they are incited we shall see that the delicacy and consequent differential value of these reactions far outstrip any known chemical methods, and it is upon this principle indeed—inexplicable as it is—that the great diagnostic value which these reactions have attained depends. The conception of the specificity of the causes of infectious disease, as well as that of the specificity of toxins, has become so common and self-evident to us that we are too apt to forget how fundamental to progress the establishment of this fact was in the early days of bacteriological research. When, in 1878, Koch published his treatise on the "Etiology of Wound Infections" specificity was not generally accepted, and the supposed metamorphosis of bacterial species, as asserted by Hallier and others,[65] had first to be scientifically refuted by Cohn, Koch, and their pupils, before it could be assumed that a given infectious

[64] Neufeld. *Deutsche med. Woch.*, No. 11, 1897. Neufeld and Rimpau. *Deutsche med. Woch.*, No. 40, 1904. Bail and Kleinhans. *Zeitschr. f. Imm.*, Vol. 12, 1912. Weil. *Zeitschr. f. Hyg.*, 75, 1913.

[65] Hallier. Cited from Behring, "Bekampfung der Infektionskrankheiten," Leipzig, 1894.

disease was always the result of infection with a definite and constant species of bacteria. The same applied to the specificity of toxins—and rational investigations into the reaction of the animal body against bacterial poisons was not possible until the works of Roux and Yersin on diphtheria and that of Kitasato on tetanus had differentiated between the true, specific bacterial poisons and the unspecific ptomains and "sepsins" of Selmi, Nencki, and Brieger.

The problem of the reasons for specificity has been and still remains one of the unsolved mysteries of immunology. We have seen in a preceding section that Abderhalden [66] in attempting to form an analogy by which the infinite variety of specific relations can be visualized, used the simple arithmetical consideration of the enormous variety of combinations that can be made by rearrangements of the twenty-one amino acids of which all proteins seem to be built up. The question, however, does not seem to be as simple as this.

In order to avoid needless repetition, we refer the reader to the section on Antigens on page 105, where we discuss in some detail the investigations of Obermeyer and Pick, and of Landsteiner and his collaborators, on the alterations produced in the specificity of proteins by the simple substitution of various inorganic chemical radicles, in various places in the protein molecule. Apparently a relatively slight chemical change by the introduction of diazo groups, iodin or bromin, or methyl radicles, in the protein molecule completely changes specific relations; and not only is the mere introduction of such groups important, but the manner and place in which they are introduced may cause further alterations. It is through researches like those of Landsteiner that a closer approach to the problem of the reason for specificity must be sought, and the infinite varieties of possible specific differences need not surprise us if we consider that some of the substances which Landsteiner introduced into the protein molecule and which completely changed its specific relations, entered only into the tyrosin and histidin nuclei of the proteins which constitute only 3 per cent. of the particular protein molecule with which the experiments were done.

Studies like those of Wells upon the chemistry of different antigenic proteins are also of considerable importance in this connection. They are dealt with in the section on Anaphylactic Antigens, particularly.

[66] Abderhalden, *Münch. med. Woch.*, 14, 1913.

CHAPTER IV

THE MECHANISM OF NATURAL IMMUNITY AND THE PHENOMENA FOLLOWING UPON ACTIVE IMMUNIZATION

ANTIGENS AND ANTIBODIES. THE ORIGIN OF ANTIBODIES

Pasteur's work on active immunization was carried out in the later seventies and the early eighties. During and immediately after this time it was very natural that the attention of investigators should have concentrated upon the elucidation of the causes underlying both the natural resistance against bacteria observed in animals and man, and the changes which during active immunization were fundamentally responsible for the acquisition of resistance.

It was easily determined that there were no anatomically and physiologically determinable differences between the various mammalia which could account for the observed striking variations of susceptibility, nor could gross anatomical or histological changes be noted in an animal which had been artificially immunized. Morphologically such an animal was indistinguishable both in the size and appearance of its organs, and in the arrangement and structure of its cells from any other individual of the same species not subjected to treatment.

It was a natural development of the investigations brought to bear upon this problem that attention should, for a time, be concentrated upon the phenomena of inflammation, processes which were regularly associated with infections of all kinds and seemed indeed to represent a sort of local expression of tissue resistance to the invading micro-organisms.

It was in the course of investigations upon the nature of inflammation that Metchnikoff first became interested in problems of resistance. In 1883 he presented a paper at the Naturalists' Congress at Odessa, in which he referred the absorption of dead or foreign corpuscular elements in the bodies of invertebrates to a process of intracellular digestion carried out by phagocytic cells. As early as 1874 Panum had suggested that possibly resistance against invading micro-organisms might be due to a similar intracellular destruction, and Metchnikoff, soon after his first communication, extended his phagocytic studies to phenomena of infection. His first investiga-

tions concerned themselves with an infectious disease caused by a form of yeast in a small crustacean—the daphnia or water flea. He showed that recovery or death from the disease depended upon the completeness with which the invading micro-organisms were taken up by the white blood cells found in the body cavity of the daphnia. Immediately subsequent studies, carried out with the aid of numerous pupils, embraced an extensive material throughout the animal kingdom in which he attempted to show parallelism between natural immunity and the phagocytic activities mobilized by the body against the invading germs.

Meanwhile studies along another path were in progress. It had been observed many years before this by the physician, Hunter, that the shed blood of animals was not as easily subject to putrefactive change as were many other organic substances. Similar observations by Traube and, in 1881, by Lord Lister [1] (the latter reported at a time when Pasteur's experiments were reaping their first practical results) further stimulated investigation of the blood as the possible seat of the increased antibacterial property. For, indeed, these observations seemed to imply that by resisting decomposition, even when inoculated with putrefying material, the blood must possess definite means of inhibiting or even destroying the putrefactive bacteria.

In 1884, in a dissertation submitted at Dorpat, Grohman [2] stated that cell-free blood plasma inhibited the growth of micro-organisms. But Grohman was unable to determine actual bacterial destruction. Similar, but inconclusive, observations were published by Von Fodor [3] in 1887. In 1888, however, Nuttall,[4] who was investigating the validity of the phagocytic theory of Metchnikoff, experimentally determined that normal blood possessed the property of killing bacteria—a property now spoken of as "bactericidal" power. The attitude taken by Nuttall, and others of the Flügge school, toward Metchnikoff's opinions was one of doubt as to the fundamental significance of phagocytosis in determining resistance. They argued that Metchnikoff had not yet proved that *living* bacteria were taken up by the phagocytic cell, and that the action of these cells might therefore be interpreted as merely a process of removal of the dead bacteria, after these had been killed by other influences. Nuttall, accordingly, repeated some of Metchnikoff's experiments on anthrax in frogs and rabbits, essentially confirmed the basic observations, but showed also that the cell-free defibrinated blood of these and other animals possessed definite bacteria-destroying properties (bacteri-

[1] Lister. *Trans. Intern. Med. Congress,* London, 1881.
[2] Grohman. Cited from Lubarsch, *Centralbl. f. Bakt.,* 6, 1889.
[3] Fodor. *Deutsche med. Woch.,* No. 34, 1887.
[4] Nuttall. *Zeitschr. f. Hyg.,* Vol. 4, 1888.

cidal power) for many different micro-organisms. He detected similar properties in pleural exudates, pericardial fluids, and aqueous humor, and determined that this property was "inactivated" or destroyed when the fluids were heated to 55° C. for 10 minutes or longer. Buchner [5] then confirmed Nuttall's results and showed further that the bactericidal property resided, not only in defibrinated blood, peptone blood, and plasma, but was present also in the serum obtained after clotting. He applied the term *"Alexin"* to this active constituent of the blood—likening its action to that of a ferment.

The immediate theoretical result of these discoveries was an attempt, begun by Flügge's school, to base natural as well as acquired resistance upon the bactericidal properties of the blood and body fluids in general. For the observations of Nuttall and Buchner were soon extended to peritoneal and other exudates by Stern,[6] and to ascitic fluids by Prudden.[7] By these two groups, that of Flügge-Nuttall-Buchner on the one hand, and that of Metchnikoff on the other, there were founded the two schools of immunity—the humoral and the cellular, both originating in attempts to explain natural immunity, and later extending to problems of acquired resistance. And it is to the diligent and ingenious intellectual and experimental conflict between these schools that we owe much of the knowledge we now possess concerning the phenomena of immunity. A bridge between them was early established when Buchner himself—(even before Metchnikoff)—suggested the possible leukocytic origin of the bactericidal serum constituent (alexin). The later work of Denys, of Gruber and Futaki, of Wright, of Neufeld, of Bail, and of others has demonstrated, as was to be expected, the inadequacy of either point of view by itself, and the intimate interdependence of the humoral and the cellular processes.

As concerns the relation of bactericidal serum effects and natural immunity, it could be unquestionably shown by Nuttall, Buchner, Nissen,[8] and their immediate followers that the blood of most animals possessed bactericidal properties against many micro-organisms, their experiments being so planned that the participation of leukocytes could be absolutely excluded. However, a parallelism between bactericidal power and the degree of natural resistance could not be established. Lubarsch,[9] writing during the early periods of the controversy, stated that "he would regard the (purely humoral) [10] experiments of Nuttall as decisively contradicting the phagocytic

[5] Buchner. *Centralbl. f. Bakt.,* 1889.
[6] Stern. *Zeitschr. f. klin. Med.,* Vol. 18 (cited from Hahn).
[7] Prudden. *Med. Rec.,* Jan., 1890.
[8] Nissen. *Zeitschr. f. Hyg.,* Vol. 6.
[9] Lubarsch. *Centralbl. f. Bakt.,* Vol. 6, 1889.
[10] Bracketed phrase our own.

theory if the bactericidal action of the blood (for anthrax bacilli) could be shown to be more potent in immune than in susceptible animals." Metchnikoff [11] himself, taking this point of view, called attention to the fact that the blood serum of rabbits, animals that are highly susceptible to anthrax, is more powerfully bactericidal for these micro-organisms than is the blood of dogs or even that of immunized calves, both of which are much more resistant than are rabbits. Nuttall answered this by reporting that the blood of anthrax-immunized calves is actually more powerfully bactericidal than is that of normal calves. Although this argument of Nuttall was perfectly valid in principle, it exerted little influence on opinions at this time because anthrax happens as a matter of fact to belong to that group of infections in which bactericidal protection is actually secondary to phagocytic, and Lubarsch could show that the differences observed by Nuttall were often less than those obtaining between specimens of blood taken from individual normal rabbits.

Lubarsch himself, then, in carefully planned experiments, showed that rabbits and cats could be killed with quantities of anthrax bacilli far less than the number which the extravascular blood of these animals can destroy. He concluded that the resistance, in these cases at least, is certainly not parallel with the bactericidal properties of the blood, and suggested the possibility that the intravascular blood does not possess bactericidal power to the same degree in which it is possessed by the extravascular plasma or serum. This point, first raised by Lubarsch—namely, the possibility of a difference between the intravascular blood and the extravascular blood serum or plasma in bactericidal functions—soon became one of the focal points of the controversy, since Metchnikoff, admitting the bactericidal power of the shed blood, assumed that this was purely the result of substances given off by the leukocytic cell-bodies after extravascular injury.

The Metchnikoff school defended its premise by the dual method of attempting on the one hand to establish a parallelism between phagocytic activity and natural resistance, and, on the other hand, by showing that the cell-free blood serum of naturally resistant animals often furnished an excellent culture medium for the bacteria in question. Thus Wagner showed that anthrax bacilli grow well in the blood of fowls at 42° C., and Metchnikoff himself called attention to the fact that pigeons' blood is an excellent medium for the cultivation of the Pfeiffer bacillus, whereas the living pigeon is entirely insusceptible to influenzal infection. Arguments based on such observations, however, have lost much of their original weight, for we have since then learned more about the delicate quantitative conditions and the difficulties of accurate measurements obtaining in

[11] Metchnikoff. *Virch. Archiv,* Vol. 97, 1884.

experiments upon *in vitro* bactericidal phenomena. For although a specimen of the blood of a naturally immune animal may be capable of destroying a considerable number of bacteria of a given species, the implantation of such a specimen with a slight excess of the bacteria would soon exhaust the active serum constituents and profuse growth could then take place. Furthermore, the conditions of temperature established in cultural experiments lead rapidly to a deterioration of the alexin necessary for bactericidal action, and any bacteria remaining alive at the end of a number of hours would then have unopposed opportunity to multiply.

The attempts to establish parallelism between phagocytic activity and natural immunity, though somewhat more successful than the analogous efforts of the humoral school, nevertheless also failed to furnish complete explanation for existing conditions, and, as we shall see, no adequate generalizations could be made until later years revealed the close coöperation between cells and fluids. We must postpone any attempts to do justice to this phase of the problem, therefore, until we are in a position to discuss the question of phagocytosis on the basis of a fuller knowledge of the phenomena which influence it.

In the chapter on phagocytosis we will see that experiments by Kyes and others have shown that the natural immunity of some animals (birds) to pneumococci can be largely explained by phagocytic activity on the part of such fixed tissue cells as the Kupfer cells of the liver. It is not unlikely that in all processes of natural immunity the phagocytosis by tissue cells is an important protective factor.

The clear thinking and unprejudiced logic brought to bear upon this controversy by some of the great bacteriologists of this time are nowhere more instructively illustrated than in a short introduction published by v. Behring [12] to his second article on diphtheria. He says: "Neither deduction nor theorizing can at present decide whether a compromise will be found in the future between the two hypotheses (humoral and cellular), or whether the one or the other alone will be found correct. As yet the opinions of many experimenting bacteriologists are in direct opposition in this respect. Meanwhile, for the purposes of medical advancement and therapeutic success it is not necessary to await a decision of this question. . . . It is indeed of advantage to the cause if the struggle against infection is undertaken from the most varied points of view; attempts to make proselytes for a dogma have never led to progress. In this sense I will try to summarize those experimental results which support the humoral point of view without attempting particularly to detract from the importance of opinions which I do not share."

[12] v. Behring. *Zeitschr. f. Hyg.*, Vol. 12, 1892.

THE PHENOMENA FOLLOWING UPON ACTIVE IMMUNIZATION

The cellular and humoral points of view, formulated largely upon the facts of natural immunity, were equally applied, almost from the beginning, to the explanation of active immunization. The light thrown upon these phenomena by the efforts of both schools rapidly led to a complete abandonment of those earlier theories of immunity which had conceived the acquired resistance of animals against bacteria as a purely passive development in the body of conditions unfavorable for bacterial growth.

Among these earlier theories, now of historical interest only, are the "Exhaustion Theory" of Pasteur and the "Retention Theory" of Nencki,[13] Chauveau, and others.

Pasteur's views, defended for a time also by Garré, held that the growth of any given variety of bacteria in the animal body exhausted certain specific nutritive substances necessary for this growth. Subsequent lodgment in the same body was impossible owing to the absence of proper nutrient material. It is interesting to note, as Kolle [14] points out, that this theory is in principle very similar to the "Atrepsie" idea of Ehrlich advanced in explanation of species immunity to cancer.

The hypotheses of Chauveau, of Nencki, and others were the converse of those of Pasteur. They were based purely on inference, assuming that conditions occurring in the test tube could be applied also to those existing in the animal body. Baumann [15] had shown that, among other things, phenol was produced as a result of bacterial putrefaction. Nencki had noticed the inhibition of bacteria in culture by the products of their own metabolism. Wernicke,[16] too, had demonstrated the presence of phenol, phenylacetate, skatol, and other aromatic compounds harmful to bacteria in putrefying mixtures. The reasoning which formulated the so-called "Retention Theory," therefore, was the following: Bacteria growing in the animal body produce certain substances peculiar to their own metabolism, which eventually lead to inhibition of their growth. By the retention of these products the animal is rendered immune. Chauveau's adherence to this theory was largely based on the fact that he had observed immunity in the lambs born of Algerian ewes which had recovered from anthrax shortly before or during parturition. He explained this by a transference of the retention products from

[13] Nencki. *Jour. f. Prakt. Chem.*, May, 1879, cited from Sirotinin, *Zeitschr. f. Hyg.*, Vol. 4, 1888.
[14] Kolle in "Kolle u. Wassermann Handbuch," 2d Ed., Vol. 1.
[15] Baumann. *Zeitschr. f. physiol. Chem.*, Vol. 1.
[16] Wernicke. *Virch. Archiv*, Vol. 78.

mother to offspring. As a matter of fact the observation could just as well have been utilized as support for the Exhaustion Theory.

Both the theory of "Exhaustion" as well as that of "Retention" could not long withstand experimental criticism. Theories which were not so easily disproved and which have given rise to much investigation are the "Alkalinity Theory," first formulated by v. Behring,[17] and the "Osmotic Theory" of Baumgarten.[18] In the former an attempt was made to demonstrate a parallelism between blood alkalinity and bactericidal action—the latter was based on the supposition that the destruction of bacteria in the body was largely due to harmful osmotic conditions. Neither of these theories was long seriously maintained. Behring himself took an active part in the subsequent development of our present views. Baumgarten [19] still clings to his own opinion in a modified way, in that he maintains that the only effect produced by specific antibodies upon cells—bacterial or otherwise—is that they change the permeability of the cell membranes and render them more vulnerable to osmotic injury.

However crude or vague these theories may seem to us now, it must not be forgotten that they were conceived at a time when no knowledge had been gained regarding specific "antibodies." The phagocytic powers to which Metchnikoff attributed natural immunity and the bactericidal powers of the blood, regarded in the same light by the Flügge school, were general properties possessed by many animals toward many different micro-organisms. That immunization could specifically increase these functions toward the particular micro-organisms used for treatment seemed indicated by the experiments of Nuttall in which higher bactericidal power was found in the blood of anthrax-immune calves than in that or normal animals. However, no definite and conclusive work on the specific increase of measurable serum or cell properties was available.

This great advance, giving new energy and pointing out new paths of investigation, came in 1890-1892 with the publication of the work of Behring and his collaborators, Kitasato and Wernicke, on immunity to diphtheria and tetanus. As we have indicated in a preceding paragraph, the fundamentally important points of this work were as follows:

1. The establishment of the fact that animals may be actively immunized with products of bacterial metabolism—true toxins or exotoxins.

2. The discovery that such active immunity was dependent upon specific antibodies formed in the treated animal and circulating freely in the blood; and,

[17] v. Behring. *Centralbl. f. klin. Med.*, 1888, No. 38.
[18] Baumgarten. *Berl. klin. Woch.*, 1899, 1900.
[19] Baumgarten. *Lehrbuch, etc.*, 1912.

3. That, by the transfer of the blood or the blood serum containing these specific antibodies, other normal animals could be passively protected—not prophylactically only, but even after active disease had set in.

These observations were rapidly confirmed for tetanus by Tizzoni and Cattani, and by Vaillard, and, similar but less successful attempts at passive immunization were made in other diseases by Foa, Emmerich, Bouchard, and many others. The discovery of passive immunization established the fact of specific alteration of the blood by active immunization, and represented, for the time, a distinct triumph for the humoral hypothesis.

Summarizing the knowledge of immunity as it stood at the close of this period, Behring says: "In the case of natural immunity no generally applicable explanation has as yet been found. (By this he referred to the lack of complete parallelism between natural immunity and either the bactericidal or the phagocytic activities.) For artificial immunization, however, it has now been shown, in a number of carefully studied infections, that we can surely attribute it to properties of the cell-free blood."

Within a very short time after Behring and Kitasato's first paper Ehrlich [20] demonstrated that the principle discovered by them was not limited to bacterial poisons. He was investigating immunization against ricin in mice, and showed that here, too, the blood of the immune animals contained a body which would antagonize the toxic action of ricin, and which, injected into normal mice, would passively protect them. He spoke of this blood constituent as "antiricin."

It is natural that extensive generalization followed these discoveries. However, while it was found that the blood of all actively immunized animals possessed a certain degree of protective power for normal individuals, it was soon shown that this was not due in all cases to antagonism to the bacterial poisons on the part of the immune blood serum. In immunity to the Vibrio Metchnikovi—in pneumococcus and cholera immunity—Sanarelli,[21] Isaeff,[22] Pfeiffer and Wassermann,[23] and a number of others showed that here, unlike diphtheria and tetanus, the protective power of the immune serum did not rest on "antitoxic" properties, but rather on antagonism to the bacteria themselves. It soon became definitely established that antitoxic immunity resulted only in the cases of those bacteria in which a true soluble exotoxin was produced, and where the disease following infection was primarily due to the absorption of these poisons. The antibodies incited in the blood of toxin-immune

[20] Ehrlich. *Deutsche med. Woch.*, No. 32, 1891.
[21] Sanarelli. *Ann. Past.*, Vol. 7, 1893.
[22] Isaeff. *Ibid.* and *Zeitschr. f. Hyg.*, Vol. 16, 1894.
[23] Pfeiffer and Wassermann. *Zeitschr. f. Hyg.*, Vol. 14, 1893.

animals were therefore spoken of by Behring and Ehrlich as "*antitoxins*" and their action—after a number of false hypotheses—was finally recognized as a direct neutralization of the bacterial poisons.

The strict specificity of these antibodies was, from the first, clear to v. Behring, who observed that diphtheria-immune serum and tetanus-immune serum acted each upon its respective toxin only. It was recognized at the same time that the passive immunity produced by injecting antitoxic sera is almost immediately established; that, by proportionately increasing the amount of antitoxin, immunity can be produced against any amount of toxin; and that this passive or transferred immunity is of relatively short duration.

The antitoxins, then, as we shall see in the more detailed analysis of their action (in Chapter V), are specific poison-neutralizing antibodies formed in the blood of animals immunized with a true bacterial toxin or exotoxin—conferring resistance or immunity, not by influencing the bacteria, but by rendering innocuous the specific bacterial poisons.

The therapeutic successes of passive immunization achieved with tetanus and diphtheria very naturally led to a careful inquiry into the antitoxic properties of the blood of animals immunized with all known pathogenic bacteria and bacterial products, and with many poisons of animal and vegetable origin.

Contrary to earlier expectations, however, the list of bacteria against which antitoxic immunity can be achieved has remained relatively small, limited in fact, as we have previously stated, to those species which produce a soluble exotoxin. The inciting of a specific neutralizing antibody (antitoxin), however, is also a property of many other substances of proteid nature which are for this reason classified biologically with the true toxins or exotoxins. In fact, the one absolutely constant attribute which defines our conception of the "true toxins" and the substances classified with them is their antitoxin-inciting power. We classify a bacterial product as a "toxin" or "exotoxin" only if it incites a neutralizing "antitoxin" in the serum of an immunized animal.

The first discovery of a non-bacterial antitoxin-stimulating substance was, as we have stated, that of ricin by Ehrlich,[24] 1891, and this was soon followed by similar determinations for abrin and robin—other vegetable poisons. In 1894 Calmette,[25] and Physalix and Bertrand [26] extended the principle to poisons of animal origin by demonstrating antitoxin formation against snake poison. And that similar specific neutralizing bodies were formed in response to

[24] Ehrlich. *Deutsche med. Woch.*, 1891; *Fortschr. d. Med.*, 1891, 1897.
[25] Calmette. *Ann. Past.*, Vol. 8, 1894.
[26] Physalix and Bertrand. *Compt. rend. de la soc. de biol.*, 1894.

immunization with ferments was shown in 1900 by Morgenroth.[27]

The more important individual substances which may be biologically grouped together because of their property of inciting a specific antitoxin (or toxin-neutralizing body) in the blood of immunized animals may be tabulated as follows:

Diphtheria toxin—(*loc. cit.* Behring & Wernicke).
Tetanus toxin—(*loc. cit.* Behring & Kitasato).
The Toxin of the Bacillus of Symptomatic Anthrax—(Grassberger & Shattenfroh, *Münch. Med. Woch.*, 1900, 1901 and 10 c. cit.).
The Toxin of the *Bacillus Botulinus*—Kempner, *Zeitschr. f. Hyg.*, Vol. 26, 1897).
The Toxin of the Welch Bacillus (Bull & Pritchett *loc. cit.*).
The Toxin of the Vibrion Septigue (Weinberg & Seguin *loc. cit.*).
The Toxin of the *Bacillus Pyocyaneus*—(Wassermann, *Zeitschr. f. Hyg.*, Vol. 22, 1896).
The Toxin of the *Dysentery Bacillus* (?) Shiga-Kruse type—(Kraus u. Doerr, *Wien. klin. Woch.*, 1905).
The leukocyte poison of the *Staphylococcus pyogenes aureus, Leucocidin*—(Denys & Van de Velde, *La cellule*, 1895).
The Hemolytic Poisons of Various Bacteria (see Pribram in "Kraus und Levaditi Handbuch," Vol. II, p. 223).
Proteolytic Ferments of the Hog Cholera Bacillus (De Schweintz, *Medical News*, 1892).
The Toxin of the *Cholera Spirillum* (?) Brau & Denier, *Compt. rend. de l'acad. des sc.*, 1906, Kraus, *Centralbl. f. Bakt.*, 1906, and *Wien. klin. Woch.*, 1906).
Ricin—(Ehrlich, *loc. cit.*).
Abrin—(Ehrlich, *loc. cit.*).
Krotin—(Ehrlich, *loc. cit.*).
Snake venom—(Calmette, *loc. cit.*).
Spider poison—(Sachs, "Hoffmeister's Beiträge," 1902, and Ehrlich, "Gesammelte Arbeiten," etc.).
Lab. enzyme—(Morgenroth, *loc. cit.*).
Pepsin—(Sachs, *Fortschr. d. Med.*, 1902).
Trypsin—(Achalme, *Ann. Past.*, 1901).
Leukocytic ferments Leukoprotease—(Jochmann & Müller, *Münch. med. Woch.*, 1906).[28]

The period of investigation which was initiated by the discovery of the specific antitoxins was replete with efforts to determine true toxins and, consequently, antitoxic immunity for all pathogenic bacteria. We have already mentioned that in many cases these efforts were futile—the bacteria in question being found to secrete no exotoxin and the immunity established against them developing without the formation of demonstrable antitoxin. Metchnikoff [29]

[27] Morgenroth. *Centralbl. f. Bakt.*, 26, 1899.
[28] This list includes all the important antitoxin-inciting substances. For a more complete tabulation see Wassermann in "Kolle u. Wassermann Handbuch, etc.," Vol. IV, 1st Ed., p. 498. Our own list is adapted from the one there given.
[29] Metchnikoff. *Ann. Past.*, 1892.

showed this to be the case with hog cholera as early as 1892, and the investigations of Sanarelli, Isaeff, and Pfeiffer and Wassermann pointed in the same direction.

Perhaps the clearest definition of the conditions prevailing during immunization of animals with non-toxin-forming bacteria was that formulated at this time by Pfeiffer. The importance of the bactericidal power of serum, as discussed before this by Flügge, Nuttall, and others, had dealt largely with variations of this general property in relation to natural immunity, but had failed to recognize clearly a specific increase in these powers during active immunization. Pfeiffer with Wassermann [30] had studied the pathogenicity of cholera spirilla for guinea pigs, and had come to the conclusion that the animals died of toxemia (and not of bacteriemia, as claimed by Gruber and Wiener), and that this toxemia was due to the liberation of poisons from the dead bodies of cholera vibrios, killed by the serum of the infected animals. Pfeiffer [31] now showed that the injection of cholera spirilla killed with chloroform brought about a toxemia identical with that following inoculation with living cultures. He further determined that the resistance of animals against cholera was due to the bactericidal effects of the serum, which killed the injected cholera spirilla, and not to any poison-neutralizing property.

Isaeff,[32] one of Pfeiffer's pupils, continuing this work, expresses his own and Pfeiffer's conceptions as follows: "Guinea pigs vaccinated against cholera, in spite of high immunity to infection with living spirilla, do not develop any immunity to cholera [endo] [33] toxins. The blood of immunized guinea pigs possesses no antitoxic properties. The maximal dose of cholera 'toxin' which immunized guinea pigs can withstand is not higher than that which can be borne by normal animals, and but slightly higher than the maximal dose of living spirilla, which they can survive. The blood of cholera-vaccinated guinea pigs possesses strong specific protective powers. The same specific immunizing properties are demonstrable in the blood of cholera convalescents toward the end of the third week of the disease."

The path was thus cleared for a definite conception of cholera immunity, and this was formulated, in their next communication, by Pfeiffer and Isaeff.[34] [35] [36] In this paper they showed that the cholera spirilla injected into the peritoneum of a cholera-immune

[30] Pfeiffer and Wassermann. *Zeitschr. f. Hyg.*, Vol. 14, 1893; also Pfeiffer, *Zeitschr. f. Hyg.*, Vol. 16, 1894.

[31] Gruber and Wiener. *Archiv f. Hyg.*, Vol. 15, 1893.

[32] Isaeff. *Zeitschr. f. Hyg.*, Vol. 16, 1894.

[33] Bracketed word our own.

[34] Pfeiffer and Isaeff. *Zeitschr. f. Hyg.*, Vol. 17, 1894.

[35] Pfeiffer. *Ibid.*, Vol. 18, 1894.

[36] Pfeiffer and Isaeff. *Deutsche med. Woch.*, No. 13, 1894.

guinea pig were subjected to a rapid dissolution, a process which could be observed by taking small quantities of exudate out of the peritoneum, at varying intervals, with capillary pipettes. No such dissolution occurred in normal pigs or with normal serum. But the same rapid swelling, granulation, and, finally, dissolution occurred when the spirilla were injected into the peritoneal cavity of a normal guinea pig, together with the serum of an immunized animal. The process took place apparently without the coöperation of the leukocytes or other cells, and was absolutely specific. For instance, no "lysis" occurred when the vibrios "Nordhafen," "Massauah," and other cholera-like organisms were injected into cholera-immune pigs, but took place regularly when true cholera strains, from various sources, were used in the experiment. The immunity of cholera-treated animals, therefore, was found to be an antibacterial and not an antitoxic one. Cholera spirilla introduced into a normal animal were permitted to multiply and accumulate until a sufficient number were present to furnish, upon cell death, a fatal dose of poison. In immunized animals the small quantities of bacteria first introduced succumbed rapidly to the lytic properties of the serum and accumulation was prevented.

By these experiments, now commonly spoken of as the "Pfeiffer Phenomenon," it was definitely proved that active immunization with bacteria incites in the serum of the treated animal a potent increase of bactericidal properties—an increase which is entirely specific in that the bactericidal power toward bacteria other than those employed in the immunization does not exceed the normal. The immunity in these cases, then, is not *antitoxic,* but rather *"antibacterial,"* and depends on the development, in the immune sera, of antibodies quite distinct from the *"antitoxins."* These immune serum constituents were spoken of by Pfeiffer as *"bacteriolysins"* or "specific bactericidal substances."

Not long after the discovery of the specific bacteriolysins another property of immune sera was described by Gruber and Durham.[37] They had been studying bacteriolytic phenomena with colon and cholera organisms, and noticed that these bacteria were rapidly agglomerated and gathered in small clumps when emulsified in homologous immune serum. Similar clumping had indeed been described before. Metchnikoff, Isaeff, Washburn, and Charrin and Roger had described it on various occasions, but had not recognized it as a specific property of immune serum.[38] Gruber and Durham studied it carefully, determined that it was present to a degree roughly proportionate to the degree of immunization attained, and that its specificity was such that it could be utilized for bacterial differen-

[37] Gruber and Durham. *Münch. med. Woch.,* 1896.
[38] For references see chapter on Agglutinins.

tiation. They believed that the substances in the immune serum responsible for this agglutination were independent of other serum constituents and applied to them the term *"agglutinins."*

The problems of immunization had now considerably expanded and the nature of the new serum reactions was assiduously studied. Primarily the phenomenon of agglutination was regarded as a part of the struggle of the body against the living bacteria and Gruber himself believed that it depended upon a swelling or "klebrig werden" of the microörganisms which tended to cause their sticking together, and rendered them more readily amenable to the action of the bactericidal powers of the serum. Bordet,[39] however, early conceived the process as a physical phenomenon in which the bacteria themselves were entirely passive, and, indeed, Widal [40] soon demonstrated that bacteria killed by heat were equally as agglutinable as the living germs.

This naturally suggested that the reaction between specific agglutinating serum and bacteria was based on individual peculiarities of the bacterial proteins, and it occurred to Kraus,[41] accordingly, to investigate whether or not the immune sera would cause any sort of reaction when mixed with the dissolved body substances of homologous bacteria. Working at first with cholera and plague, he prepared solutions of bacterial proteins, both by allowing broth cultures to stand for varying periods and by emulsifying agar cultures in alkaline broth. The extracts were then filtered through Pukal filters to remove the bacterial bodies. When the sera of immunized animals were added to these clear filtrates—cholera serum to cholera filtrate, and plague serum to plague filtrate, slight turbidity developed and was followed within twenty-four hours by the formation of small flakes. In other words, it was found that the mixture of a clear filtrate of a bacterial culture with the serum of an animal immunized against these bacteria resulted in the formation of a precipitate. The reaction was found to be as strictly specific as that of agglutination.

Although, from the beginning, Paltauf [42] attempted to associate the phenomena of agglutination and precipitation, the property of precipitating homologous culture filtrates was attributed by Kraus and others to specific antibodies in the immune sera, distinct and independent of those previously described, and spoke of them as *"precipitins."*

The discovery of the various "antibodies" so far discussed resulted from the study of the direct action of blood serum upon

[39] Bordet. *Ann. Past.*, 1896.

[40] Widal. *La semaine médicale*, No. 5, 1897.

[41] Kraus. *Wien. klin. Woch.*, No. 32, 1897.

[42] Paltauf. "Discussion of Kraus' Paper," *Wien. kl. Woch.*, No. 18, 1897, p. 431.

bacteria and bacterial products. This did not, however, completely deflect the attention of investigators from the unquestionable importance of phagocytosis in the defence of animals against bacterial invasion. Metchnikoff and his school continued diligently to pursue this other phase of the study of immunity and, although the increasing knowledge of serum antibodies continued to strengthen the premises of the purely humoral point of view, it had still to be admitted that in some diseases—particularly anthrax and the pyogenic coccus infections, phagocytosis must largely be held responsible for recovery. It was found, moreover, by the later investigations of Denys, Wright, Neufeld, and others that phagocytosis in immunized animals was far more extensive and efficient than in normal ones, and that this depended on specific constituents of the immune serum which rendered the bacteria more amenable to the phagocytic action of the cells. These further antibodies we will discuss in a subsequent chapter, under the terms *"opsonins"* and *"bacteriotropins,"* designations applied to them by their discoverers.

We have thus reviewed briefly the various specific properties which develop in the serum of an animal when it is systematically treated (actively immunized) with bacteria or bacterial products. These serum activities have been attributed to the development in the serum of substances which we speak of as *"antibodies."*

In our discussion of the first of these antibodies, antitoxin, we call attention to the fact that the principle discovered in the case of bacterial toxins was rapidly extended to vegetable poisons, snake venom, spider poison and enzymes. It was found that the power of inciting antitoxins when injected into animals was an attribute belonging to a large group of substances in nature, and not limited to bacteria alone. A similar generalization of conception has been possible with other antibodies. Specific lysins, agglutinins, and precipitins may be produced by the treatment of animals with many substances not of bacterial nature.

The first observation of this kind was made almost simultaneously by Bordet [43] and by Belfanti and Carbone.[44] They observed that the serum of an animal that had been treated with the red cells of another species acquired the power of laking these cells. That the normal serum of one species is often toxic to, and causes the laking of, the erythrocytes of another species is an observation that dates back to the earliest experiments on transfusion, and had been studied in considerable detail by Landois as early as 1875. The phenomenon possesses much interest in its bearing on the problems of anaphylaxis and will be discussed more particularly in that connection. We mention it in this place to show that, like bactericidal bodies, "hemolytic" (erythrocyte laking) properties may be present in nor-

[43] Bordet. *Ann. Past.*, Vol. 12, 1898 .

[44] Belfanti and Carbone. *Giorn. della R. Acad. di Torino,* July, 1898.

mal sera, though irregularly and by no means occurring in every species of animal. Incidentally it may be stated that this is true also of agglutinins and of opsonins which may be found in considerable amounts in normal sera. Of precipitins, however, this does not seem to be true.

By the work of Bordet it was found that "hemolysins" could be specifically [45] incited in an animal by systematically treating it with the red blood cells of another species. Apart from the great interest attaching to this discovery in itself, it has had a very profound influence upon investigations on immunity generally, since it has furnished a method of studying lysis far more simple and easily controlled than is the analogous phenomenon of bacteriolysis. And since, in fundamental principles, bacteriolysis and hemolysis are essentially alike, much of our knowledge regarding the former has been arrived at by experiments upon the latter. The specific *hemolysins,* then, are antibodies formed in response to "immunization" with red blood cells, analogous to the similarly produced "bacteriolysins." Because both of these antibodies exert definite injury upon cells, we speak of them by the group names of *"cytolysins"* or *"cytotoxic"* substances.

The discovery of hemolysins naturally suggested the use of other cells, and the following years brought forth many reports of further specific cytotoxins. In 1899, Landsteiner,[46] and very soon afterward Metchnikoff,[47] described specific *"spermotoxins"* which appeared in the blood of animals treated with spermatozoa. Von Dungern [48] obtained analogous substances by injecting ciliated epithelium from the trachea. Neisser and Wechsberg [49] produced *"leukotoxin"* by injecting leukocytes; Delezenne [50] produced *"neurotoxin"* and *"hepatotoxin,"* and Surmont,[51] *pancreas cytotoxin.* Subsequent years have added to these *"gastro-toxin"* (Bolton),[52] *thymotoxin* (Slatineau),[53] *adrenal cytotoxin* (Gildersleeve),[54] *placentar cytotoxin* (Frank),[55] *corpus luteum cytotoxin* (Miller),[56] and a number

[45] By the use of the word specific in this case we imply that an animal immunized with any given variety of red blood cells will form hemolysins for this variety only. Thus an animal treated with ox blood will form ox blood hemolysins only, and his serum, though strongly hemolytic for ox blood, will not lake sheep cells, dog cells, human cells, etc.

[46] Landsteiner. *Centralbl. f. Bakt.,* Vol. 25, p. 549, 1899.

[47] Metchnikoff. *Ann. Past.,* Vol. 13, 1899.

[48] Von Dungern. *Münch. med. Woch.,* p. 1228, 1899.

[49] Neisser and Wechsberg. *Zeitschr. f. Hyg.,* Vol. 36, 1901.

[50] Delezenne. *Ann. Past.,* 1900; *Compt. rend. de l'acad. des sc.,* 1900.

[51] Surmo nt. *Compt. rend. de la soc. de biol.,* 1901.

[52] Bolton. *Lancet,* 1908.

[53] Slatineau. Cited from Roessle, *loc. cit.*

[54] Gildersleeve. Cited after Roessle.

[55] Frank. *Jour. Exp. Med.,* 1907.

[56] Miller. *Centralbl. f. Bakt.,* 47, 1908.

of others. In fact, as Roessle[57] puts it, in a review of the literature, there is no organ in the body for which it has not been claimed that specific cytotoxins can be formed by the injection of homologous macerated tissues.

Recent critical study of these organ-cytotoxins has revealed, however, that the specificity of a serum produced with the tissues of one organ is not strictly limited to this organ alone, and that the serum may injure other organs as well. It is true, indeed, that there are certain cells and tissues in the body such as the spermatozoa, the tissues of the testicles, the ovary, the lens of the eye, and, possibly, the placenta which have chemical characteristics so well defined and individual that the cytotoxic sera induced by them have definite organ specificity. The same to a more limited extent seems true of kidney substance (Pearce). In most cases, however, in which originally a specific cytotoxin was claimed, it has been possible to show subsequently that the apparently selective injury was due not to organ specificity alone but to the fact that the injection of tissue-macerates, even when sufficiently freed from blood, induced the formation of considerable amounts of hemagglutinins and hemolysins.

Pearce[58] expresses it as follows: ". . . it is evident that the cells or the various organs of the body, while differing in morphology and function, have certain (receptor) characteristics in common, and that one type of cell may therefore produce antibodies affecting several cells of differing morphology, but with like (receptor) groups. This is shown by the sera prepared from washed liver, kidney, pancreas, and adrenal, all of which may agglutinate and hemolyze red blood cells and may cause degenerative changes also in the liver and the kidneys. Some of these cytotoxic sera have no effect upon organs for which they are supposed to have a morphological affinity, but exert a powerful lytic influence upon other cells. Aside from nephrotoxin, which has a distinct injurious action upon renal epithelium, the various cytotoxins studied (kidney, liver, pancreas, and adrenal) have no specific action in the morphological sense."

This opinion seems to be in harmony with that of most observers who have studied the problem recently, at least as regards most of the organ cytotoxins. Much of the promised light upon pathological processes—looked for when cytotoxins were first studied, has faded, moreover, since it has been found that cytotoxins cannot be produced by injection into an animal of cells, tissues, or fluids from its own body. "Autocytotoxins" in general cannot be produced, a question discussed at greater length in the chapter on lysis, in connection with Ehrlich's work on the isolysins.

The work outlined in the preceding paragraphs had thus ex-

[57] Roessle. "Lubarsch und Ostertag," Vol. 13, 1909.
[58] Pearce. *Jour. of Med. Res.*, N. S., Vol. 7, 1914, p. 13.

tended the principles of antitoxin and lysin production beyond the scope of pure bacteriology, and had shown them to possess the significance of general biological laws. Similar generalization was soon attained in the case of the agglutinins and in that of the precipitins. In the former, the nature of the reaction limited it to observations upon cells in suspension, and, in connection with the earlier experiments upon hemolysis it was soon discovered that the erythrocytes were often clumped before lysis could take place, when brought together with a hemolytic serum of moderate or feeble potency, or when solution, for other reasons, was delayed.

The first observations on the general significance of the precipitin reaction we owe to Tschistovitch [59] and to Bordet.[60] Tschistovitch was studying the toxic action of eel serum upon rabbits. This serum, as Kossel [61] had shown, is toxic for rabbits and possesses the property of causing hemolysis of rabbit erythrocytes. Its similarity to ricin, in this respect, stimulated attempts to produce an antitoxic substance against eel serum, even as Ehrlich had produced an antiricin. In the course of such experiments Tschistovitch observed that, when eel serum was mixed with the serum of a rabbit which had received several injections of this substance, the mixture became rapidly opalescent and soon a flocculent precipitate was formed. Coincident with this discovery Bordet made a similar observation. He had injected chicken blood into rabbits in the course of experiments upon hemagglutination. He found that the serum of the rabbits so treated acquired the property not only of producing hemolysis and hemagglutination of chicken cells, but also of giving a precipitate if mixed with chicken serum.[62] Soon after this precipitins were produced by injecting rabbits with milk (Bordet), egg albumen (Ehrlich, Uhlenhuth), and many other substances, and the specificity of such reactions was demonstrated by Fish,[63] Wassermann and Schütze,[64] Uhlenhuth, and many others.

It is apparent from the preceding paragraphs that the discovery of specific antitoxins merely constituted the first step in the formulation of a fundamentally important biological law. There is, then, a large group of substances of animal and vegetable origin which call forth the formation of specific reacting bodies when injected into animals. In order to elicit this response it is necessary that these substances shall penetrate to the physiological interior of the

[59] Tschistovitch. Cited by Bordet, *loc. cit.*, and also *Ann. Past.*, 13, 1899.

[60] Bordet. *Ann. Past.*, Vol. 13, 1899.

[61] Kossel. *Berl. klin. Woch.*, No. 7, 1898.

[62] This, we know now, was due to the fact that the blood cells injected were not washed free of chicken serum. Thus chicken serum precipitin was formed as well as were hemagglutinin and hemolysin.

[63] Fish. *St. Louis Med. Cour.*, 1900. Cited from Uhlenhuth.

[64] Wassermann and Schütze. *Deutsche med. Woch.*, No. 30, 1900. Vereinsbeilage.

body in a relatively unchanged condition. For this reason any form of injection, subcutaneous, intravenous, or into a serous cavity, is followed, with regularity, by antibody formation, whereas feeding or other means of intraintestinal administration is negative in result, unless abnormal conditions prevail which permit entrance into the blood before the digestive enzymes have decomposed the ingested materials.

The substances with which antibody-formation may be induced are collectively spoken of as *"antigens."*

The Conception of the Antigen.—Antigens are all substances which, injected into the animal body, induce specific antibody formation. They form a large group in nature and are chemically proteins; indeed, we may say that all known proteins may act as antigens. Whether or not this term may also include lipoid-protein combinations, lipoids or the higher protein derivatives is as yet uncertain and need not in the present connection concern us.

We may divide antigenic substances into two main classes.[65] One of these comprises all of those substances of bacterial, animal or vegetable origin which, injected into the animal body, give rise to specific *neutralizing* or *antitoxic* properties in the blood of the injected animal. These are the bacterial exotoxins, the snake venoms, some powerful vegetable poisons and proteolytic and other enzymes of animals and plants. They are all substances which are powerfully active—some of them strongly toxic to the living animal, others true enzymes or ferments. Indeed all of them possess properties which at least suggest our placing them into the class of enzymes in general. The number of such substances known is limited. The reaction they call forth in the animal body seems aimed directly at the specific neutralization of their respective activities, and is so unique and different from that induced by other antigens that it would be convenient had we another term like *"antitoxinogen"* to set them apart by themselves.

The other class of antigens comprises all proteins which are inactive, showing in themselves neither toxic nor enzyme-like properties. Introduced into the animal body parenterally, they call forth a response of a nature entirely unlike that of the antitoxins, and which as far as we can fathom its purpose seems aimed merely at the assimilation or the removal of the infected substance. For the cells of the animal cannot utilize the foreign protein as such, and thus it is only foreign proteins injected into an animal that act antigenically, and no antibodies are formed when homologous material is injected.

This large group, composed of all formed and unformed sub-

[65] See also Zinsser, "The More Recent Developments in the Study of Anaphylactic Phenomena." *Arch. of Int. Med.,* Vol. XVI, 1915, pp. 223-256. Harvey Lecture.

stances in nature in which a protein structure is involved, does not induce the formation of anything like the neutralizing antitoxins spoken of above. The antibodies appearing in animals treated with such substances have been spoken of as cytolysins or cytotoxins—precipitins—and in the case of formed antigens like bacteria or blood cells—agglutinins and opsonins. As we shall see in another section, it is our opinion that all these various antibodies are identical in structure and significance.

We must not forget, however, that the observation of antibodies in the circulating blood is but one of the changes that have taken place in the treated animal. Much has been made of this phase of the problem because serum antibodies are readily studied in vitro; but their origin of course must be sought in the body cell, in which the original and most profound changes must necessarily have taken place during such treatment, changes the nature of which are to a large extent still a mystery, but on which ultimately depend the important physiological difference between treated and untreated animals. For such changes—whether we refer to those immediately under discussion, namely, those of allergy or anaphylaxis, or whether we think of the so-called immunity remaining after attacks of many diseases—remain present long after the circulating antibodies have disappeared and must therefore be regarded as associated with profound alterations in the ultimate tissue unit, the body cell.

A good deal of our knowledge of the chemical nature of antigens has been developed recently, since the more delicate anaphylactic reactions have been available. For it is often possible to demonstrate antigenic properties by the anaphylactic experiment when the more gross determinations in the test tube are too weak for definite interpretation. There are certain chemically true proteins which have no antigenic properties. The most important of these is gelatin. Starin [66] found that the chief difference between gelatin and other proteins consisted of a deficiency in the gelatin of tryptophan and tyrosin, and a low content of phenylalanine. Wells [67] believes it possible that the deficiency in aromatic amino acids may be responsible for the lack of antigenic power. Wells and Osborne [68] have found, on the other hand, that zein from corn lacks glycin and tryptophan. Gliadin of wheat and hordein of barley contain neither glycin or lycin, and very little arganin or histidin. These proteins are powerfully antigenic, and yet extremely poor in diamino acids. Wells suggests that since this is the case, and since, conversely, there are protamines which are poor antigens and consist chiefly of diamino acids, that the diamino acids are of little or no importance as antigens.

[66] Starin. *Jour. Infec. Dis.*, 23, 1918, 139.
[67] Wells. *Phys. Rev.*, 1, 1921.
[68] Wells and Osborne. *Jour. Infec. Dis.*, 8, 1911, 66.

The question of the antigenic properties of protein cleavage products has been largely investigated, and in general we may say that the products of protein hydrolysis have not been found antigenic. Zunz [69] and, more recently, Fink [70] working in Wells' laboratory, have given this subject particular attention. Zunz produced heteroalbumose and protoalbumose by peptic and tryptic digestion of fibrin, and claims that he was able to sensitize guinea pigs and rabbits to the anaphylactic reaction with these substances. Attempts to repeat his work by Friedberger and Joachimoglu [71] were unsuccessful and the amounts which it was necessary for Zunz to use in the second injections in his guinea pigs and rabbits made it impossible to exclude the possible primary toxic action of his albumoses. Moreover, we know that many of the protein-split products injected into animals give rise to symptoms not at all unlike anaphylactic reactions. Thus, Zunz's work is not decisive. Hailer [72] studied the digestion products of beef muscle, chiefly, with negative results. When he did get reactions, they were lacking in specificity and were always extremely feeble. Fink, working with the cleavage products of hydrolyzed egg white, found slightly antigenic properties in fractions precipitated at three-quarters and complete saturation with ammonium sulphate, but not for those obtained with lower concentrations of the salt. Results of all these investigators which suggest possible antigenic properties of such cleavage products may well be explained by Wells' [73] observation that beef serum digested as long as 16 months with trypsin, did not completely lose all of its coagulable material, in spite of the fact that the Biuret reaction had disappeared. It is, therefore, extremely difficult to be sure that there was not a certain amount of whole undigested protein left in the preparations used by the observers mentioned.

Investigation of the antigenic properties of altered proteins has led to a considerable amount of important information which eventually may prove to clear up the antigen question. Of great importance is Ten Broeck's [74] discovery that when protein is racemized with alkali by Dakin's method and is, in consequence, so changed in configuration that it is optically altered and no longer susceptible to enzyme action, it loses its antigenic properties.

Perhaps the most interesting and important progress in this line of work is that initiated by Obermeyer and Pick.[75] These investigators altered proteins by various chemical methods. They distin-

[69] Zunz. *Zeit. f. Immunitats.*, 16, 1913, 518.
[70] Fink. *Jour. Infec. Dis.*, 25, 1919, 97.
[71] Friedberger and Joachimoglu. *Zeit. f. Immunitats.*, 24, 1914, 522.
[72] Hailer. *Arb. a.d.k. Gesamtsht.*, 527, 1914.
[73] Wells. *Jour. Infec. Dis.*, 6, 1909, 506.
[74] Ten Broeck. *Jour. Biol. Chem.*, 17, 1914, 369.
[75] Pick. *Biochemie der Antigene,* Jena, Fischer, 1912; Kolle & Wassermann Handbuch, *Wien. Klin. Woch.*, 22, 1903, 10, 1904, 12, 1906.

guished between methods which simply changed physical constitution and others which actually interfere with the chemical structure of the protein. Heating, the action of toluol, chloroform, simple acids and alkalis, produced modifications of antigenic properties, but did not alter species specificity. This is in keeping with certain investigations upon the cocto-precipitins made by Schmidt and by ourselves, which are discussed in the precipitin chapter. However, when they introduced NO_2 groups by treatment with nitric acid or diazotized the protein with nitrous acid, or iodized it by treatment with Lugol's solution, they found that the specificity of the antigenic properties of the protein was actually altered. Thus, an immune serum produced by the treatment of animals with any one of these altered proteins, no longer reacted with the native horse serum from which it had been produced, but reacted strongly with similarly altered proteins from other species. The species specificity, therefore, had given way to a specificity of chemical structure. Since in these reactions the change produced was due to some substitution in the aromatic radicals of the proteins, they suggest that such changes are of paramount importance to antigenic function. This would fall into agreement with Wells' suggestion that the deficiency in aromatic amino-acids is responsible for the lack of antigenic properties found in gelatin, and fortify the conclusions drawn by Wells and Osborne from their very careful structural studies of antigens, that chemical structure is the important factor since chemically similar gliadin and hordein, even when derived from different plants, may be antigenically very similar to each other. Landsteiner [76] with many collaborators, has given this subject a considerable amount of attention. It is quite impossible in the confines of this book to go into the details of all the important and laborious work which Landsteiner has published on this subject. We must, therefore, select from all his experiments, a few to illustrate their work. One of the processes used particularly was diazotizing the proteins. An example of their procedure as given by them, is as follows: 100 c. c. of horse serum were treated with 100 c. c. of normal soda solution, and a small amount of a 1 per cent. solution of diazo-benzol produced from analin was added. The solution was cooled and a drop tested for alkalinity with phenolphthalein. Then it was allowed to stand in ice-water for about 10 minutes and precipitated with hydrochloric acid. The precipitate was caught on a filter paper, taken up in a little water and brought into solution by the gradual addition of normal soda solution and then precipitated with alcohol. This precipitate was again dissolved in water, and again precipitated

[76] Landsteiner. *Zeit. f. Immunitäts.*, Vol. 20, 1913, 211 and 618; Vol. 21, 193; Vol. 26, 136; *Biochem. Zeit.*, Vol. 58, 362, 61, 67, 74, 86, 1918, 343 and 93, 1919, 106; *Proc. Royal Academy of Sciences,* Amsterdam, 25, No. 1, p. 34.

with alcohol, and this again taken up in water by the addition of a small amount of soda solution. It was then neutralized to litmus, and brought to a volume of about 200 c. c. in 1 per cent. salt solution to which ½ per cent. phenol had been added. The alcohol precipitation was employed in order to remove toxic substances which were found to be present unless this was done, and in some cases this had to be repeated in order to permit injection into animals. For further particulars, we refer to Landsteiner's paper in the *Biochemische Zeitschrift* for 1918, page 343. The general conclusions of his work may be stated as follows: The specificity of antigens is apparently determined by the chemical structure of relatively small parts of the large antigen molecule. This is particularly evident from the fact that as Pauli [77] has shown, the diazo bodies, introduced among other things by Landsteiner, enter only the tyrosin and histidin groups of protein, which would constitute about 3 per cent. of the proteins used. In the course of this work, Landsteiner did a large number of reactions with 23 different cases or antisera produced with 33 different azo-proteins. Six of the immune sera reacted only with antigens which had been produced in a homologous way, in regard to the azo components. In some of the others there were group reactions. Obermeyer and Pick's observation that the species specificity was entirely altered, and a chemical specificity now substituted, was definitely confirmed, and it was further shown that the particular place in the aromatic ring in which the new radical, that is the azo-group was introduced, was also important in determining specificity.

With such slight chemical differences determining antigenic properties, it is not at all impossible that the differences in the species specificity of animal proteins may depend purely on structural variations in the aromatic amino groups, which, because of the isomeric nature of proteins, it would be almost impossible to define with chemical exactitude at the present time.

Moreover, such investigations also make it quite easy to understand that the same serum or protein of any kind is a mixture of a number of different and separately specific antigens. This has been often found, and especially emphasized in the work of Dale and Hartley [78] who separated the euglobulin, pseudoglobulin and albumoses of horse serum, and found that each fraction could be made to sensitize separately. Similar work has been done in the separation of protein constituents of egg albumin.

Before leaving the subject of antigens it seems advisable to add a brief discussion of a form of substance which is antigenic only in the sense that it reacts specifically with antibodies, but with which the formation of antibodies by injection into the animal has not yet

[77] Pauli. *Zeit. f. Physchem.*, 42, 1904, 512, Vol. 94, 284 and 428.
[78] Dale and Hartley. *Biochem. Jour.*, 10, 1916, p. 110.

been demonstrated. Some years ago, Landsteiner suggested that tuberculin might be such a substance, largely because, in the case of this material, specific reactions are produced in the injected animal body, but attempts to produce antibodies with it have failed. Our own later researches have lent strong probability to this suggestion of Landsteiner. From a considerable number of bacteria, tubercle bacilli, pneumococci, staphylococci, influenza bacilli, meningococci, we have been able to obtain substances which remain in the residue after boiling with acid, and an attempt to remove all of the ordinary protein materials. These substances are precipitable with alcohol, easily soluble in water, and in extremely minute amounts give powerful reactions with anti-sera, both precipitating and fixing alexin specifically. Although our work is not finished, we have not been able to produce antibodies with these materials when sufficiently purified.

In view of the chemical studies detailed above, it seems quite likely that the antigenic function of specific union with the antibody may be dependent upon a relatively small nucleus of the protein molecule, which perhaps we have been able to split off by the methods used by us. Permitting ourselves a tentative speculation, our work suggests to us a teleological conception of antibodies in the following way: Antibodies are formed in the animal body only upon injection of entirely non-diffusible substances like the true proteins. These substances being of large molecular size and non-diffusible, can, therefore, react with the cellular elements of the body only by cell surface relations, and in order to go into reaction with the tissues, antibodies are necessary and, therefore, formed. Since the molecule becomes smaller, and relation with the cell by a certain amount of diffusion becomes possible, antibody formation becomes less and less necessary as a physiological reaction. In a vague way this conception associates antibody formation with molecular size. These considerations are still largely speculative, but are rapidly taking form in experimental work which we have not been able to finish at the present writing.[79]

For substances such as those described above, namely, with the ability to unite with antibodies, but the apparent lack of ability, possibly based on small molecular size, of stimulating antibody formation in the animal body, Landsteiner has proposed the term *Haptene*.

Since the phenomenon of antibody formation is not at all limited

[79] It must not be supposed in this connection that the function of antibodies in the disposal of foreign proteins or bacteria can be regarded as a process of direct ingestion. Proteolytic and other enzyme activities have not been shown to be involved in the reaction between antibodies and their antigens, whether or not alexin took part in these reactions. This has been particularly investigated by Jobling and Petersen. See *Jour. A. M. A.*, 65, 1915, 515 and 66, 1916, 1753; *Archiv. Inter. Med.*, 15, 1915, 286.

to bacteria or bacterial derivatives, it cannot be looked upon merely as a mechanism existing for the primary purpose of protecting the body against infectious disease. This latter function is important, indeed, but the process undoubtedly has a much more general biological significance.

In the course of normal existence substances which are not directly assimilable as such—foreign proteins, for instance—do not penetrate directly into the blood and tissues. Taken into the alimentary canal, they are first hydrolized into peptons, albumoses, polypeptids, and probably amino-acids before absorption, to be reconstructed from these cleavage products ("Bausteine" is Abderhalden's expression for the amino-acids) into protein biologically identical with that of the tissues. Digestive and other accidents, however, on numerous occasions during life permit the direct entrance of these materials unchanged or insufficiently changed into the circulation. It is probably by the action of digestive powers of the serum—or, in the case of the entrance of the undissolved foreign particles, by the activity of the phagocytic cells—that such substances are then disposed of and assimilated. For each particular variety of substance (antigen) a specific mechanism is called into play, and when this mechanism is repeatedly called upon—as in successive injections of foreign proteins—this mechanism, whatever it may consist of, is enhanced in efficiency—i. e., increased in quantity. How this increase of specific antibodies is theoretically conceived we will discuss later in connection with Ehrlich's side-chain theory.

The phenomena of antibody formation against bacteria on this basis may be taken to constitute, then, a mechanism for the disposal [80] of a foreign protein which has penetrated into the tissues and, because of its living state, increases within the body by multiplication, furnishing progressive stimulation to the antibody-producing function. Infectious disease, therefore, from this point of view may be looked upon as an invasion of the body by a living foreign protein which must be assimilated and disposed of; which, in some cases, has a primary toxicity per se; and which is variously distributed among the organs and tissues according to the biological peculiarities of the particular micro-organism in question. This general conception will become more clear as we analyze the phenomena associated with the individual antibodies. It is, of course, quite plausible as far as it refers to the phagocytic functions, or even bacteriolytic and cytolytic phenomena. It has been less clear in connection with the agglutinins and precipitins in which a direct defensive or bacteria-destroying value is not apparent. However, in our discussions of these phenomena we will have occasion to point out many reasons for assuming that, even in these phenomena, there are

[80] It will be seen in a later section that the action of antibody and alexin on an antigen does not imply proteolysis or any form of digestion.

features which fall into direct correlation with the views we have just expressed.

The substances which possess antigenic properties—that is, which give rise to antibody production—with the exception of a few isolated and contested cases, are all of them protein in nature. Well-trained chemists have exerted themselves to purify antigenic substances, in attempts to determine the particular fractions of the complex protein molecule upon which the antigenic properties depend. In the course of such work a number of men claim to have obtained a truly antigenic substance which no longer gave protein reactions. The instance most frequently cited is Jacoby's [81] announcement of a protein-free ricin. Jacoby worked with an apparently very impure "Ausgangsmaterial" consisting of commercial ricin, which he digested for five weeks in trypsin solution. At the end of this time he obtained a ricin which still possessed the properties of the original castor-bean extract, but no longer gave protein reactions. His "purified ricin," however, was quickly destroyed by further trypsin digestion, and more recent work by Osborne, Mendel, and Harris [82] appears to have fully refuted Jacoby's results. They found the purified ricin identical with the coagulable albumin of the castor bean, and found that tryptic digestion destroys the characteristic ricin properties.

Less easily refuted have been the careful experiments of Ford [83] upon the active principle of a mushroom (*Amanita phalloides*) and upon that of the poison-ivy plant—(*Rhus toxicodendron*). These substances, he claims, are non-protein. In the case of *Amanita phalloides* Abel and Ford [84] have shown it to be a glucosid, and similar structure has been claimed for Rhus by Syme.[85] Yet with both of these substances Ford has succeeded in producing specific antitoxins. Rabe [86] has questioned these with *Amanita phalloides.* He believes that the poison with which Ford worked is not a glucosid, but is of protein nature. In the case of Rhus, however, Ford's conclusions have not, to our knowledge, been challenged.

With these and a few other less important exceptions, however, observers have uniformly concluded that antigenic property and protein structure are inseparably associated. All procedures by which proteins have been hydrolized into their simpler fractions, chemical splitting, tryptic or peptic digestion have in every case resulted in a simultaneous loss of protein reaction and antigenic property.

We will see in a later discussion of relationship of antigenic structure to anaphylactic reaction that the possibility has been sug-

[81] Jacoby. *Arch. f. exp. Path. u. Pharm.*, Vol. 46, 1901.
[82] Osborne, Mendel, and Harris. *Am. Jour. of Physiol.*, 1905, Vol. 14.
[83] Ford. *Jour. Inf. Dis.*, Vol. 3, 1906; Vol. 4, 1907.
[84] Abel and Ford. *Jour. Biol. Chem.*, 1907.
[85] Syme. *Johns Hopkins Thesis*, 1906.
[86] Rabe. *Zeitschr. f. exp. Path. u. Therap.*, Vol. 9, 1911.

gested by Landsteiner and by us, that the antigenic nature of the proteins may be not only a question of chemical structure, but also one of molecular size, our own idea being that the antibody mechanism is a provision by which it is made possible for the body to deal with substances that cannot diffuse into the cell substance.

Many attempts have also been made to show a relation between antigenic properties and the lipoid constituents of cells. These endeavors were obviously stimulated by the observation that many lipoids are capable of binding antibodies *in vitro,* and that, in nervous tissues, toxin fixation was in some way related to the richness in lipoids of these structures. Bang and Forsmann [87] accordingly treated animals with ether extracts of red blood cells—claiming that this resulted in the production of hemolysins. And these results have been confirmed by Landsteiner and Dautwitz.[88] The latter, however, suggest that the hemolysin production may have been induced, not by the lipoidal substances in solution, but by other antigenic substances which had gone into colloidal suspension in the ether extracts. Much similar research on the antigenic nature of lipoids has been done, but, after reviewing this very thoroughly, Landsteiner comes to the conclusion that no definite proof of the antigenic nature of any pure lipoid has so far been presented. The problem is experimentally complicated by the fact that, as Landsteiner [89] suggests, the antigen may often be present as a lipoid-protein combination, and as such go into solution or fine emulsion in the organic solvents; also the lipoids possess the curious property of altering the solubilities of proteins and other substances by their presence.

Summarizing our present knowledge of the chemical nature of antigens, then, we must conclude that, with the exception of Ford's glucosids, no protein-free antigens have been thus far demonstrated.

In the light of this fact it is all the more remarkable that antigen-antibody reactions are specific. For we possess no chemical methods by which one variety of protein can be distinguished from another. And yet the serum antibodies produced with each species of bacteria react with this species only—and the hemolysins, agglutinins, or precipitins produced by the injection of bacterial, cellular, or serum proteins react respectively only with the particular variety employed in their production. This indicates that each of these antigens—of almost unlimited number—must possess a chemical structure individually characteristic and different from all the others. It is by means of the biological reactions, indeed, that we can detect protein in dilutions far beyond the reaction-sensitiveness of chemical tests and can distinguish between varieties of protein when the chemical

[87] Bang and Forsmann. *Hofm. Beitr.,* 1906; *Centralbl. f. Bakt.,* 40, 1906.

[88] Landsteiner and Dautwitz. *Hofm. Beitr.,* 9, 1907.

[89] Landsteiner. "Wirken Lipoide als Antigene?" *Weichardt's Jahresbericht,* Vol. 6, 1910.

methods will indicate only protein in general. Our knowledge of the chemical constitution of protein has not yet advanced to a point at which specificity can be based upon definite variations of chemical structure, and the complexity of the problem is such that it does not seem likely that we can hope in the near future to attain such knowledge. We can merely accept it as a fact that the antibody produced with one protein differs materially from that produced with another, and that this is a definite indication that the antigen in one case must be chemically different from that in another.

The range of such variations is apparently enormous. For each variety of bacteria or plant, each species of animal, and to a certain extent each individual of the species, possesses certain special antigenic characteristics peculiar to itself. In general there is an underlying antigenic similarity which is peculiar to the species. This is true of bacteria and, in the case of animal and vegetable proteins, an antibody produced with material from an individual of a certain species will react with the protein derived from this species in general. However, that there are also antigenic differences between individuals within the same species is indicated by Ehrlich's experiments on the antibodies produced by injecting the blood cells of one goat into another. And we have further indicated that within the same animal different organs may possess individual antigenic characteristics. Added to this we know that certain special organs like the testicle, the lens, and some others contain antigens which are peculiar to this variety of organ, irrespective of species—a condition spoken of as *"organ specificity."* Thus an antibody produced by injections of the testicular substance of one animal will react with testicular protein from many different species—the specificity here depending upon the organ and not upon the zoölogical relationship.

It is clear, therefore, that there are more different varieties of protein, biologically distinguishable, than there are species of living beings in nature. As Abderhalden [90] has recently pointed out, this is a conception which it is a little difficult to grasp chemically, since in breaking up different proteins into their "building stones" (Bausteine) we encounter again and again the same 20 amino-acids. By a simple arithmetical consideration, however, he shows that merely by combining these twenty amino-acids in different groupings an enormous number of isomeric but varying compounds can be formed—even without assuming the additional possibility of quantitative variations. He reasons that 3 "Bausteine"—A, B, and C—could form 6 different structures, A B C, A C B, B C A, B A C, C A B, C B A. Similarly 4 could form 26, and finally 20 could form 2, 432, 902, 008, 176, 640, 000 different compounds.[91]

[90] Abderhalden. *Münch. med. Woch.*, No. 43, 1913.

[91] We have not repeated the arithmetical labor and take Abderhalden's word for it.

This problem is more fully dealt with in the section dealing with antigens.

Drug Tolerance.—The analogy between the active immunization of animals with the various antigens and certain chemically well-defined poisons, alkaloids, etc., is so obvious that it has led to much speculation as to a possible similarity in the physiological mechanisms of the two phenomena. As a matter of fact the acquired tolerance for such substances as morphin, atropin, and other alkaloids is not really analogous to the physiological reactions which follow the treatment of animals with bacterial and other proteins, for whatever toxic properties there are in the latter are, as we shall see later, rather the results of the interaction of these injected substances and the reaction products supplied by the cells and fluids of the body. It is at least probable in the light of our modern conception that such protein antigens are not toxic per se, in the native state. This, however, will receive detailed consideration in succeeding sections. The analogy of drug tolerance, however, to the acquired immunity against true bacterial toxins and vegetable poisons like ricin, crotin, and others is a striking one, since in both classes of poisons there is a gradually developed tolerance for substances toxic in the native state and often very similar in physiological effects (strychnin and tetanus toxin, etc.). In the case of the toxins, however, there is a development of immunity by actual neutralization of the poisonous principle brought about by a specific antibody, which circulates in the blood of immunized animals and man—the process following, within certain limits, the law of multiple proportions. In the case of morphin and other alkaloids no such neutralizing antibodies have as yet been demonstrated.[92] Whereas toxin immunity is passively transferable from one animal to another with the blood serum, and, *in vitro,* the mixture of the toxin with the immune serum brings about a neutralization of the poison, no such phenomena have been observed, as a general rule, in the case of the alkaloids. We say "as a general rule" since an exception is recorded in the observations of Fleischmann,[93] who claims to have found antagonistic action to atropin in the blood of normal rabbits, this power being absent from the blood of rabbits that had thyroid hypertrophies and were, in consequence, atropin-susceptible. Other observations of a similar significance have been made by Physalix and Contejean [94] on curare, but have not been confirmed, and the investigations of all other workers on this subject have had negative results. It seems from available evidence that

[92] Hans Meyer and Gottlieb. "Exp. Pharm.," 2d Ed., Neban & Schwartzenberg, Berlin, 1911, p. 517.

[93] Fleischmann. *Archiv. f. exp. Path. u. Pharm.,* 62, 1910, cited from Meyer and Gottlieb, *loc. cit.*

[94] Physalix and Contejean. Cited from Meyer and Gottlieb.

tolerance (immunity) against drugs is due to cellular rather than to serum antagonism. In this connection we refer the reader to the section on Drug Idiosyncrasies.

The Origin of Antibodies.—The tissue cell, as the ultimate functional unit, must, of course, be looked upon as the source from which originate the various protective constituents of normal and immune sera; and, though perhaps unrecognizable by the coarse tests of morphological investigations, it is in the cells that changes must take place primarily when the animal body is subjected to any one of the processes spoken of as immunization. The exact location of the antibody-forming cells and tissues, in spite of much investigation, is not at all clear, though many data seem to point to the lymphatic organs, the spleen, and the bone marrow as particularly concerned with this process.

Thus Pfeiffer and Marx [95] exsanguinated animals five days after injections of dead cholera spirilla and found that at this time bacteriolytic antibodies were more concentrated in the spleen than in the blood serum itself. Wassermann's [96] analogous experiments with typhoid bacilli seemed to show a higher antibody content in spleen, bone marrow, thymus, and lymph nodes than was present in the blood at an early period of immunization. Although these investigations, as well as many others of Castellani,[97] seem, therefore, to indicate a particular association of the special lymphatic organs with antibody formation,[98] extirpation of the spleen [99] before immunization has not prevented animals from responding to injections of bacteria and red blood cells with sharp antibody production. The experiments of Deutsch,[100] in which reduction of antibody formation resulted in animals in which splenectomy was practiced three or four days after immunization was begun, can hardly be accepted as a conclusion, in the writer's opinion at least, since any severe operation or interference with the normal functions of an animal during the severe physiological strain of active immunization would naturally lead to a less perfect response. That the resistance of animals and man to infection with bacteria is not noticeably diminished by splenectomy, moreover, has been variously shown. In unpublished experiments by the writer splenectomized guinea pigs showed no difference from normal animals in regard to their susceptibility to tuberculosis. And though these and similar experiments of other workers with various bacteria are not entirely devoid of interest, their negative results as a matter of fact have no great significance,

[95] Pfeiffer and Marx. *Zeitschr. f. Hyg.*, Vol. 27, 1898.
[96] Wassermann. *Berl. klin. Woch.*, p. 209, 1898.
[97] Castellani. *Zeitschr. f. Hyg.*, Vol. 37, 1901.
[98] Pfeiffer and Marx. *Loc. cit.*
[99] I. Levin. *Jour. Med. Res.*, Vol. 8, 1902.
[100] Deutsch. *Ann. de l'Inst. Past.*, Vol. 13, 1899.

since our knowledge concerning the true function of the spleen is very incomplete, and it is not impossible that on removal of this organ other elements of the lymphatic system may take over its function in part or as a whole.

Removal of the spleen has not been an extremely unusual procedure in surgery, and there is no evidence to show that patients so treated have been abnormally susceptible to infection thereafter.

Yet, as we have seen, there seems to be an early concentration of antibodies in the lymphatic organs in the course of immunization, and it may well be that an association between the process and these tissues exists which cannot be experimentally demonstrated with absolute certainty.

It is no less likely, however, that similar functions are exerted by the cells of other organs. In fact, it is more than probable that antibodies may be formed anywhere in the body—and that the locality of their production is largely dependent upon the locality in which the antigen is concentrated. Wassermann and Citron [101] demonstrated this by injecting typhoid bacilli into rabbits intraperitoneally, intravenously, and intrapleurally, and nine days afterward determining the comparative bactericidal strength of blood serum and of aleuronat exudates of pleura and peritoneum in each of the three animals. Their results showed that the bactericidal titre of the intravenously inoculated animal was highest in the blood serum, while that of the intraperitoneally and intrapleurally inoculated animals was highest in peritoneal and pleural exudates respectively. Such experiments point to the possibility of a "local" immunity, that is, a production of antibodies directly by the cells with which the antigen comes into contact in the most concentrated and direct manner. And, indeed, another isolated experiment of the same authors, alone successful of a series of similar attempts, would point in the same direction. Typhoid bacilli were injected subcutaneously into the ear of a rabbit and the ear immediately ligated at its base and kept so for several hours. After nine days the bactericidal titre of the blood serum was determined and the ear amputated. An immediate and rapid drop of antibody contents occurred after the amputation—indicating that the chief source of antibody function had been removed. More striking examples of the same thing are to be seen in the experiments of Römer,[102] who instilled abrin into a rabbit's eye and found that the retina of the eye developed an antitoxic power against abrin which protected mice against many times the fatal dose, while that of the other eye remained practically inactive.

From these facts, as well as from other observations, it is at least reasonable to believe that antibody formation is by no means a func-

[101] Wassermann and Citron. *Zeitschr. f. Hyg.*, Vol. 50, 1905.
[102] Römer. *Arch. f. Ophthal.*, 52, 901.

tion of special organs and that many cells throughout the body may take part in the process. It is of especial importance to consider this in connection with the possible effects of the treatment of infections by means of bacterial vaccines. If the focus of the infection can possibly become also a local source of antibody production, then such treatment may well seem rationally founded, even in generalized acute infections in which no logical basis for such treatment would exist, were the production of antibodies a task for specialized organs like spleen and bone marrow only. The therapeutic phases of this problem are more extensively considered in a later chapter.

It is in this fact also that we must seek the explanation of the apparent local immunity which occurs in certain infections of the skin. Thus it frequently happens that successive crops of boils may afflict different parts of a patient's skin—new ones arising as old ones heal, showing that the process of the limitation and healing of the infected foci is not due to any increase of generalized resistance, but rather to local causes. In the same way, in erysipelas, the process extends along the edges while the original central area of infection is returning to the normal state, and it rarely occurs in adults that the erysipelatous process extends back into the originally infected area.[103] From these localized laboratories of antibody formation, of course, distribution to the circulation probably takes place and the complete cure of the patient must await a sufficient concentration of these in the body as a whole before further local foci cease to arise.

That the fixed tissue cells of any part of the body can and do take an active part in the local reaction against the invasion of bacteria and other foreign materials is histologically evident. When a more or less insoluble foreign body—a thread of lint, paraffin, agar-agar, or other material—is deposited in the subcutaneous tissues anywhere in the body, and is accompanied by acute infection with bacteria, there is a characteristic tissue reaction which results in the surrounding of the foreign particle by multinucleated cells spoken of as giant cells. In the case of foreign bodies such as those mentioned the process is purely one of local ingestion of the particle which later, if the material remains absolutely insoluble, results in encapsulation by connective tissue. If soluble, however, there may be an eventual digestion of the foreign material by the cell with a subsequent degeneration or splitting up of the giant cell and a return to normal. This also occurs in the case of such infections as those due to yeasts or blastomyces, in which, as the writer has seen, the apparent lack of liberation of toxic products gives rise to a purely local giant-cell reaction, adjacent tissue cells remaining undegenerated and apparently unaffected. In the case of infection with bacteria like the bacillus of tuberculosis, the leprosy bacillus, that of

[103] In children erysipelas not infrequently returns within a few days over a recently healed area

rhinoscleroma, and a few others the purely local picture of giant-cell phagocytosis is complicated by secondary reactions arising probably from the liberation of toxic products from the living or dead invaders which both stimulate specific cell reactions and call forth cell degeneration in adjacent tissues, frequently giving the individual infection a diagnostically characteristic appearance.

CHAPTER V

TOXIN AND ANTITOXIN

THE REACTION BETWEEN TOXIN AND ANTITOXIN (EHRLICH'S ANALYSIS)

THE TOXIN-ANTITOXIN REACTION

When Behring and his collaborators, Kitasato and Wernicke, had definitely shown that the cell-free blood serum of animals immunized with tetanus and diphtheria toxins respectively possessed the power to protect other animals of the same and different species against the poisons, it became of the utmost importance to determine, if possible, the mechanism by which the "antitoxic" effect was attained. The earlier opinion, expressed by Behring himself, held that in all probability the toxin was directly injured or destroyed by the action of the antitoxic serum. That this assumption was incorrect was soon demonstrated by the experiments of Roux and Vaillard [1] and by those of Buchner.[2] The work of the former investigators showed that the mixtures of tetanus toxin and antitoxin, measured in such proportions that they were harmless for normal guinea pigs, could still be found toxic for animals weakened by preliminary inoculation with other bacteria. Buchner claimed in analogous experiments that similar mixtures, harmless for mice, could still show toxicity for guinea pigs. He inferred from this that the nature of the cell reactions of different animal species influenced the antitoxic effect. Both investigations led the workers to conclude that the protective action of antitoxin was not due to a direct effect upon the poison but was potent by acting upon the tissue cells of the animal by protecting these from subsequent harm by the toxin. Their conception implied an indirect protective function on the part of the antitoxin, not due to any direct reaction between it and the poison.

That this explanation, too, was faulty was made evident by a number of investigations which took advantage of the peculiar differences in resistance to temperature between certain toxins and their specific antitoxins.

In 1894 Calmette [3] and Physalix and Bertrand [4] had indepen-

[1] Roux and Vaillard. *Ann. de l'Inst. Past.*, 1894.
[2] Buchner. *Münch. med. Woch.*, p. 427, 1893.
[3] Calmette. *Compt. rend. de la soc. de biol.*, 1894.
[4] Physalix and Bertrand. *Compt. rend. de la soc. de biol.*, 1894.

dently succeeded in obtaining an antitoxin against snake poison. In the course of further study of these bodies Calmette [5] determined that the venoms of certain varieties of snakes, the naja and cobra, would remain potent even when subjected to 100° C. for a very short time. In contrast to this the antitoxins to these poisons were destroyed at much lower temperatures. Now when mixtures of the two substances, so proportioned that their injection into animals was innocuous, were heated to 68° C. for considerable periods, toxic properties again became evident, a demonstration that the toxin had not been destroyed, but had remained neutral only in the presence of the intact antitoxins. These experiments were confirmed by Wassermann,[6] who found that similar conditions prevailed in the combination between pyocyaneus toxin and antitoxin.

The filtration experiments of Martin and Cherry [7] are not convincing since they may be taken as indicating either neutralization or toxin destruction. These workers subjected mixtures of snake poison and its specific antitoxin to filtration through gelatin filters, under pressure. Under the experimental conditions thus established the presumably smaller toxin molecule was allowed to pass through the filter while the larger antitoxin molecule was held back. They showed that if filtered soon after the ingredients have been put together most of the toxin still passes through, but that, as this interval is prolonged, less and less comes through, presumably because of the union of the smaller toxin to the larger antitoxin molecule. The chief value of these experiments lies in their proof of the element of time as an important factor in the toxin-antitoxin union.

In his experiments on snake venom just recorded, Calmette interpreted the restitution of toxicity after the heating of neutral mixtures of cobra neurotoxin and its antitoxin as evidence "qu'il ne s'était pas formé aucune combinaison de ces deux substances ou que la combinaison realisée était, au moins, très instable." Later experiments of Martin and Cherry seemed for a time to contradict this conclusion. Observations by them, analogous to those of Calmette, but carried out with the poison of an Australian snake, seemed to indicate that when the toxin and antitoxin were allowed to remain together for a sufficiently long time no restitution of toxicity could be obtained by heating. Apparently the application of heat to such mixtures merely prevented the further union of antitoxin with any toxin that was not yet bound at the time that the heat was applied. Accordingly Morgenroth [8] again examined these relations and found that the addition of a small amount of hydrochloric acid to mixtures of snake poison and the antitoxin resulted in the dissociation of their union.

[5] Calmette. *Ann. Past.*, 1895.
[6] Wassermann. *Zeitschr. f. Hyg.*, 22, 1896.
[7] Martin and Cherry. *Proc. of the Royal Soc.*, Vol. 63, 1898.
[8] Morgenroth. *Berl. Klin. Woch.*, No. 50, 1905, p. 1550.

To mixtures of the venom lysin and its antitoxin, neutralized and even overneutralized so that they were perfectly innocuous to susceptible animals he added hydrochloric acid until the total concentration amounted to N/18. By this method a toxin-HCl modification was produced which was dissociated from its union with the antitoxin and was extremely resistant to heat. In such a mixture of toxin and antitoxin to which hydrochloric acid had been added, heating at 100° C. in a water bath for 30 minutes destroyed the thermolabile antitoxin and, after neutralization, undiminished toxic properties could again be demonstrated by animal inoculation.

These researches and other similar ones of Morgenroth, then, form a satisfactory confirmation of the original experiments of Calmette and seem to show, beyond possibility of contradiction, that the inhibition of harmful properties of any true toxin, after mixture with its antitoxin, does not depend upon toxin destruction. But while Calmette interpreted the facts as pointing toward a failure of union of the two substances, Morgenroth's work is not incompatible with the conception of a neutralization of one by the other in the chemical sense. These experiments of Morgenroth are of great theoretical importance moreover in that they have shown that dissociation of a toxin-antitoxin complex can occur.

The nature of such neutralizations in regard to quantitative relations, speed of action, and relative concentrations, becomes apparent partly from experiments like those mentioned above, but more especially from those carried out by Ehrlich with ricin and antiricin, experiments which were primarily planned to demonstrate that the reaction between a toxin and its antibody is a direct one, not dependent upon intervention of the body cells, as at first supposed.

It had been shown by Kobert and Stillmarck that ricin, the powerfully poisonous principle of *Ricinus communis* (castor oil bean) would agglutinate the red blood cells of a number of animals. Ehrlich recognized from the beginning how closely analogous the neutralization of ricin by antiricin was to that of diphtheria toxin by its antitoxin. The former reaction furnished him with a simple method of test tube experimentation in that the agglutinating effects of ricin upon rabbits' corpuscles could be directly inhibited by the preliminary addition of antiricin. A visible reaction was thus available, which, of course, excluded absolutely the participation of the tissue cells in the antigen-antibody neutralization, and in which careful quantitative measurements were possible.

Ehrlich [9] determined by means of this method that the neutralization was accelerated by moderate heat and by concentration of the reagents and, most important of all, that the reaction followed roughly the law of multiple proportions, characteristics, all of them, which were entirely analogous to chemical reactions in general.

[9] Ehrlich. *Fortschr. d. Med.,* Vol. 15, 1897, p. 41.

When he added 0.3, 0.5, 0.75, 0.1, etc., cubic centimeters of serum from a ricin-immune goat to constant quantities of ricin, and then added rabbit cells, the hemagglutinating properties of the ricin were inhibited in direct proportion to the amount of antiricin mixed with it. And his test tube experiments were further found to represent with much accuracy the occurrences which took place within the animal body. For, similar mixtures injected into mice were toxic in direct proportion to the balance of ricin and antiricin established in the injected material.

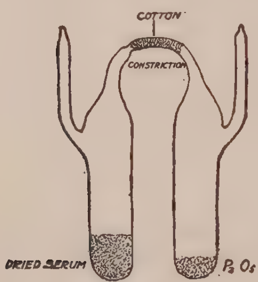
Although the views of Ehrlich and his followers have great importance in connection with the union of antigens and their antibodies in general, these ideas were worked out by him most elaborately in connection with his efforts to arrive at a practicable and accurate method of establishing a standard of strength for diphtheria antitoxin, and it is essential that we consider this work in detail in the following paragraphs.

Development of Antitoxin Standardization.—The earlier attempts to standardize diphtheria antitoxin by the use of living cultures (Roux and Behring) were soon abandoned, since it was found that the accurate establishment of fixed lethal doses of the culture was not possible. When the facts, just recorded, concerning the interaction and quantitative relations of the soluble toxins and their respective antitoxins came to light, Behring introduced the standardization of the curative sera by the use of toxins, both in the case of tetanus and in that of diphtheria. In order to do this consistently he established for diphtheria poison an arbitrary toxin unit which he defined as the amount of any given diphtheria filtrate sufficient to cause death in a guinea pig of 250 grams, and, borrowing the terms from chemical nomenclature, he designated as a "normal" diphtheria poison one which contained 100 such units in one cubic centimeter. (D T N, M250 = diphtheria toxin normal, Meerschweinchen 250 grams.)

Together with Ehrlich, Behring then established an antitoxin unit (I-E, Immunitäts Einheit). They designated as a "normal" antitoxic serum one "which contained in one cubic centimeter one antitoxic unit" (I-E), and state further, "of this serum 0.1 c. c. neutralizes 1 c. c. of the Behring normal toxin." (Conf. Madsen in "Kraus u. Levaditi Handbuch," II, p. 94.) Alterations were subsequently made in this scale of standards and Ehrlich later designated as an antitoxin unit a quantity of an antitoxin which completely neutralized 100 lethal doses (for guinea pigs of 250 grams) of a poison at that time in his possession. This definition of the antitoxin unit, however, no longer holds good and a comprehension of the true meaning of the unit can only be obtained by following in detail the methods by which it is measured. Since the methods of antitoxin standardization employed at

present in the United States were worked out by Rosenau[10] along the lines of Ehrlich's method, and the standard is based on the one introduced by Ehrlich, the antitoxin unit as employed in this country is identical with the one spoken of in the following paragraphs.

In measuring the neutralizing value of antitoxin for toxin, then, since both substances are chemically unknown and no purely chemical indicator of neutralization is available, it was necessary to select a susceptible animal by means of which excess of toxin, in mixtures of the two, could be detected. As the standard test animal guinea pigs of 250 grams were chosen, and improvements in the methods of measurement were introduced, in that the toxin and antitoxin, instead of being separately injected as heretofore, were mixed, allowed to stand for 15 to 30 minutes, and then injected together subcutaneously.



TUBE FOR THE PRESERVATION OF THE STANDARD ANTITOXIN.

Taken from Rosenau, U. S. Hygienic Laboratory Bulletin, No. 21, 1905, p. 53.

By means of this technique Ehrlich set out to examine a large number of toxins and their antibodies and obtained results which, aside from their practical value, have had an important influence upon the development of the knowledge of antigen-antibody reactions. These investigations were considerably complicated by the fact that neither the diphtheria toxin nor the antitoxin is very stable and deterioration occurs unless special methods of preservation are employed. Since the antitoxin, however, is much less unstable than the toxin, the former is employed in order to preserve the standard, and is preserved in sealed U tubes (see Figure) with anhydrous phosphoric acid. Kept in this way, in black, light-proof boxes, and at low temperature, it may be preserved for months without appreciable loss of value and may be renewed by accurate comparative measurements from time to time. This is carried out for the United States, at the present time, by the Government Hygienic Laboratories at Washington under the U. S. Public Health Service.

Preservation of the toxin is much more difficult, and it is in connection with the investigation of the instability of the toxin that Ehrlich gained his first insight into the nature of the reaction. He measured, in a number of toxic filtrates, the minimal lethal dose for guinea pigs of 250 grams, establishing a time limit for death in order to obtain more accurate comparisons. He designated as the new M L D or "T" (that is: toxic unit) the quantity of toxin

[10] Rosenau. *U. S. P. H. & Mar. Hosp. Service, Hygienic Laboratory Bull.*, 21, April, 1905.

which will kill a guinea pig of the designated weight in from 4 to 5 days. He then determined for a number of poisons the exact quantity just neutralized by one antitoxin unit, calling this quantity L_0. (L meaning Limes or threshold.)

It is clear that in judging of complete neutralization of a quantity of toxin by antitoxin, there may be a strong subjective element, since any very slight excess of toxin may cause unimportant local reactions such as edema or small hemorrhages, which could escape the attention of one observer while being noticed and recorded by another. In order therefore to exclude definitely all subjective features from such experimentation, Ehrlich now established another toxin value—L_+ dose ("Limes death"—now, for convenience, written L_+)—which eliminated all possible variations of personal perception. He designated by this symbol that quantity of toxin which not only neutralized one antitoxin unit but included enough toxin, in excess of this, to give the result of one free toxin unit, that is, to cause death in 4 to 5 days in a guinea pig of 250 grams. Since the three values just defined form the basis of Ehrlich's experiments as well as that of all practical diphtheria serum standardizations we will briefly restate them for the sake of clearness.

Thus:

M L D or "T" = the amount of toxin which, subcutaneously injected, causes death in a 250-gram guinea pig in from 4 to 5 days.

L_0 = the amount of toxin which is just neutralized by one antitoxin unit so that no trace of reaction, local or otherwise, ensues

and

L_+ = that amount of toxin which will cause death in 4 to 5 days in a guinea pig of 250 grams if injected together with one antitoxin unit.

It will further clarify the meaning of these terms to examine experimental protocols which show how these values are determined.

Thus in the following:

I. Injections of toxin
(1) .005 c. c.—guinea pig lives.
(2) .009 c. c.—guinea pig dies in 6 days.
(3) .01 c. c.—guinea pig dies in 4 days.
(4) .02 c. c.—guinea pig dies in 2 days.
.01 = M L D or T.

II. 1 Antitoxin unit + .19 toxin = late paralysis.
1 Antitoxin unit + .20 toxin = sometimes late paralysis and sometimes acute death.
1 Antitoxin unit + .21 toxin = death fourth day.
1 Antitoxin unit + .22 toxin = death in 2 to 3 days.
.21 = L_+ dose.

III. 1 Antitoxin unit + .14 c. c. toxin = no reaction.
1 Antitoxin unit + .15 c. c. toxin = no reaction.
1 Antitoxin unit + .16 c. c. toxin = slight congestion about point of injection, scarcely visible.
1 Antitoxin unit + .17 c. c. toxin = apparent reaction at site.
1 Antitoxin unit + .18 c. c. toxin = edema at site.
$L_0 = .16$.[11]

In determining these values with a large number of toxins Ehrlich discovered the curious fact that, although there was a rapid and extensive diminution of toxicity in every toxic filtrate in the course of time, there was no corresponding alteration in the L_0 amount. In other words, although more and more of the toxic broth was necessary to kill a guinea pig of standard weight in the required time, the amount of the same broth which neutralized one antitoxin unit remained approximately the same.[12]

In seeking an explanation for this apparent paradox, Ehrlich concluded that we must assume that the toxin is complexly constructed, consisting of a toxophore and a haptophore group. Assuming that chemical union between the toxin and the antitoxin (or, in disease, the body cell) takes place, it is by means of the haptophore group that such union is brought about. The toxophore group, however, is the element by which toxic action is exerted after union by the haptophore group has been accomplished. It would be conceivable, therefore, that in deteriorating in toxicity the toxin might undergo alterations in the toxophore group only, its haptophore group, and, therefore, its antitoxin neutralizing properties remaining intact. Such modified toxins, modified only as to the toxophore groups, Ehrlich now refers to as "toxoids."

In the production of diphtheria toxins for practical purposes it has been found advisable to allow them to "season," that is to stand for prolonged periods until they have reached a state of "equilibrium" at which the conversion of toxin to toxoids has been reduced

[11] II and III are taken from the article by Rosenau, *P. H. & M. H. S., Hyg. Lab. Bulletin,* 21, 1905.

[12] This statement plainly contradicts the definition of a toxin unit; i. e., the amount which neutralizes 100 toxin units and often leads to confusion among students or others who are unfamiliar with this subject. It should be borne in mind that, while the definition of an antitoxin unit is the one accepted when Ehrlich first arbitrarily established it, the antitoxin unit, as at present in use, is really an amount of antitoxin standardized against L_+ quantities of toxin, this last value again obtained by measurement against the original unit. It represents a neutralization value equal to the original one, but may protect the guinea pig against 85, 110, 130, etc. (variable), toxin units, according to the constitution of the particular toxic filtrate employed in the experiment. Indeed, if, in the following pages, the reasoning of Ehrlich is consistently adhered to, our definition of an antitoxin unit should be: The amount of antitoxin which contains 200 binding affinities for toxin. This will become clearer as the following paragraphs are read.

to a minimum and the change of relationship between L_0 and "T" or M L D has practically ceased to go on. From the very beginning of the growth of the culture in the incubator the process of toxoid formation has probably occurred, and even freshly prepared toxic filtrates therefore are not pure "toxins," especially since the conversion of toxin to toxoid seems to diminish in velocity as time goes on.

Now in spite of the presence of such alteration products, in comparing the values L_0 and L_+ of any given toxin preparation, one would naturally suppose that L_+ minus L_0 should be equal to one M L D, or the quantity just sufficient to kill a guinea pig of 250 grams in 4 to 5 days. For we have seen that L_0 just neutralizes one antitoxin unit while L_+ is the quantity which, in addition to such neutralizing power, has an excess of toxin equal in action to one minimal lethal dose. This, however, is not the case. Let us illustrate this by a concrete case. One of Ehrlich's toxins on measurement showed a minimal lethal dose or M L D of 0.0025 c. c.

	The L_+ dose of this was	.25
while	The L_0 dose of this was	.125
	The difference was	.125 or 50 M L D instead of 1 M L D

as we would suppose.

Stated in words, this measurement means that after neutralizing completely one antitoxin unit with the toxic filtrates, in order to obtain death in a guinea pig in 4 days with such a mixture, it was necessary to add, beyond the neutralizing quantity, 50 M L D, or again as much as was necessary for neutralization.

This last relation is merely coincidence, since it might have been 30 or 40 or 60 M L D just as well. The important point is the fact that more than 1 M L D was necessary, and by this fact Ehrlich was led to resort to an assumption which forms one of the basic principles of many of his explanations for serum phenomena, namely, the assumption of differences in combining avidity or affinity.

As applied to the present problem he reasoned as follows:

It is conceivable that the toxoids resulting from deterioration of toxin might possess three different degrees of affinity for the antitoxin. They might have a stronger, an equal, or a lesser affinity than the toxin itself. If their affinity for antitoxin were equal to that of toxin they would, of course, not influncee the L_+ dose itself; if stronger than toxin their influence would be so exerted that toxin would be forced out of combination with antitoxin, giving place to the toxoid, and the effect would be the opposite from that experimentally observed. If, however, their affinity for antitoxin were weaker than that of toxin each additional toxin unit added to the L_0 dose would unite with antitoxin, replacing a corresponding quantity of the toxoid of weaker affinity. In consequence, as far as the

poisonous properties of the mixture are concerned, the addition of toxin would not render the neutral mixture poisonous for guinea pigs until the toxoids had been completely displaced from union with antitoxin. Finally, after all the antitoxin had been bound to unchanged toxin, further addition would then result in the presence of free toxin and poisonous properties would again appear in the mixture. Ehrlich at first spoke of the toxoids possessing weaker affinity for antitoxin than the toxin itself as "epitoxoids." This conception can be rendered clear by the following example:

In the case cited above we had

T or M L D	= 0.0025 c. c.	
L_+	= 0.25 c. c.	
L_0	= 0.125 c. c.	
The difference	= 0.125	= 50 M L D

Supposing that the toxoids (epitoxoids) present in the mixture possessed an affinity for antitoxin less than that of toxin, the following conditions might obtain:

151 toxin-antitoxin + 49 epitoxoid-antitoxin = L_0.
Add 1 M L D or T and we have:
152 toxin-antitoxin + 48 epitoxoid-antitoxin + 1 epitoxoid free.
Add 2 M L D or T and we have:
153 toxin-antitoxin + 47 epitoxoid-antitoxin + 2 epitoxoid free until, finally, adding 50 T, we get:
200 toxin-antitoxin + 49 epitoxoid free + 1 toxin free = L_+.[13]

Later experience led Ehrlich to abandon the opinion that the epitoxoids were deterioration products of the toxin. He found that the relation between L_0 and L_+ which we have just outlined, was demonstrable in the same way, in freshly prepared toxin filtrates, in which there had been little time for toxoid formation. He further noticed that, even after deterioration had occurred to a considerable extent, and the amount necessary to kill a guinea pig had been much

[13] An example identical in significance with the one just given, but somewhat simpler in its arithmetical conditions, is here added for the sake of permitting no possibility of unclearness. This example is taken from Ehrlich's own work.

T = .01 c. c. of the toxin bouillon.

L_+	(neutral. of antitoxin unit yet killing 1 pig)	= 2.01 c. c. or 201 T.
L_0	(complete neutral. of 1 antitoxin unit)	= 1 c. c. or 100 T.
	Difference	= 1.01 c. c. or 101 T.

100 toxin-antitoxin + 100 epitoxoid antitoxin = L_0;
Add 1 T, and we have:
101 toxin-antitoxin + 99 epitoxoid-antitoxin + 1 epitoxoid free;
Add 101 T, and we have:
200 toxin-antitoxin + 100 epitoxoid free + 1 T free = L_+.

increased (the number of fatal doses in L_0 constantly decreasing as toxoids replaced toxin), the L_+ nevertheless remained unchanged. This, he held, could mean one thing only. The elements present in toxic broth which possessed a weaker affinity for antitoxin than the toxin itself, namely, the epitoxoids, were present from the very beginning and were probably separate and primary secretion products of the diphtheria bacilli, remaining relatively stable and constant as the broth was preserved. In order to avoid confusion, therefore, he now referred to the "epitoxoids" as "toxons"—to preclude their confusion with the other toxoids or true toxin deterioration products. These toxons possess, according to Ehrlich, a "haptophore" group identical with that of the toxin, but have a different toxophore group. For there is reason to believe that the toxon, lacking the power of causing acute death, gives rise to slow emaciation and paralysis, finally killing after a subacute or chronic course. Thus, in the tabulation just preceding, we have seen that the toxic broth added to neutral mixtures of toxin and antitoxin (containing the L_0 dose), does not give rise to the acutely toxic effect of one M L D or T until an amount has been added which considerably exceeds one toxin unit. This, we explained, by Ehrlich's reasoning, on the supposition that "epitoxoids" or "toxons" are displaced from their union with antitoxin, giving place to toxin and becoming free. Such toxin-antitoxin mixtures—in which the amount of toxin broth ranges between the L_0 and the L_+ doses—therefore, contain no free toxin units but contain varying amounts of free toxin. An injection into guinea pigs is not followed by acute death in these cases, but leads with considerable regularity to emaciation, paralysis, and death after a long incubation period.

It has been objected to this, as we shall see, that the slow poisoning produced by such mixtures is due, not to a qualitatively different poison but to the presence of minute quantities of free toxin too small to produce acute death, yet sufficient to produce this gradual injury. This Dreyer and Madsen [14] tried to disprove by experiments in which they prepared antitoxin-toxin mixtures so balanced that they possessed the toxon effect, and of these mixtures injected increasing multiples. In no case did they succeed in producing acute death even when the amount injected had been multiplied to such an extent that free toxin, if present, must have asserted itself. The same workers were able to show that the injection of these mixtures, in which free toxons were assumed to be present, produced immunity against toxin, thus indicating the similarity of the haptophore group of toxin and toxon—a conception which will become more and more clear as we consider the "Side-Chain Theory" which Ehrlich evolved as a result of his toxin analysis.

Ehrlich had thus elicited facts which seemed to him to indicate

[14] Dreyer and Madsen. *Zeitschr. f. Hyg.*, Vol. 37, 1901.

the presence of three qualitatively different substances in toxic filtrates of diphtheria cultures. Two of these, the toxin and the toxon, were present, he assumed, in freshly prepared filtrates, as independent primary secretion products of the bacilli, the toxin an acute poison, the toxon a substance with slower and qualitatively different poisonous effects. Both of them, toxin and toxon, possessing similar haptophore groups, could unite with antitoxin and neutralize it, but the affinity of toxon for antitoxon was weaker than that of toxin. For this reason toxin could displace toxon from its union with antitoxin, this accounting for the discrepancy between the L_+ and the L_0 doses. The third class of substances, the toxoids, were deterioration products of toxin, the deterioration implying an alteration in the toxophore group only, the haptophore group remaining the same.

It is plain from this reasoning that Ehrlich's conception implies complete analogy between chemical reactions in general and the neutralization of toxin by antitoxin. Accordingly it is but another step in the same direction to speculate concerning the actual relations of valency existing between the two substances. It seemed to Ehrlich that there were many reasons for assuming that the union between toxin and antitoxin occurred in proportions of 200 to 1; that is, just as the formula for water is H_2O, that of toxin-antitoxin combinations would be "$\text{Toxin}_{200}\text{Antitoxin}$."

The considerations on which this opinion was based were as follows: In examining a large series of toxic filtrates, Ehrlich,[15] as well as Madsen, had found that the number of toxin units ("T" or M L D) necessary to neutralize one antitoxin unit (that is, the number of toxin units contained in the L_0 dose) corresponded, with great regularity, to multiples of 100. Values of 25, 33, 50, 100, etc., recurred again and again. This indicated that the deterioration of the toxin into toxoids followed a certain regularity of progression and seemed to justify the assumption that the absolute binding power possessed by antitoxin was represented by a valency corresponding to a multiple of 100. Since the number of toxin units contained in an L_0 dose rarely fell below, and usually above 100, the valency could not be less than 100. On the other hand, repeated measurements of L_0 and L_+ doses never showed as many as 200 toxin units. Ehrlich's own highest value was 133, and the highest ever obtained by any one was a measurement by Madsen of 160. Now considering the fact that no toxin is "pure" but that, in every case, it contains admixtures of toxoid and toxon, the values 133 or 160 cannot represent all the valencies of an antitoxin unit. They represent only that part of the antitoxin unit which is neutralized by the "toxin," as measurable upon guinea pigs, a certain fraction of antitoxin being united to toxoid or toxon. It is likely, therefore, as Ehrlich reasoned, that, being higher than 100, and in an ob-

[15] Ehrlich. *Deutsche med. Woch.*, No. 38, 1898, Vol. 24.

viously impure condition approaching but never reaching 200, the valency of antitoxin for toxin was just 200. The correctness of this surmise seemed rendered more probable by Ehrlich's further studies, since analysis of the ingredients of various toxic filtrates, that is, the determination of the relative contents of toxin, toxoids, and toxon, appeared to show constantly definite relations to the valency 200.

The method by which Ehrlich carried out these subsequent studies is spoken of as *the method of "Partial Absorption."* In principle it represents a reversal of his earlier methods of measurement. In these he had titrated various amounts of toxin broth against a constant quantity (one unit) of antitoxin, establishing the L_+ and L_0 values. In the method of Partial Absorption, on the other hand, he measured varying fractions of an antitoxin unit against a constant amount of toxin, employing for this a previously determined L_+ and L_0 dose. A measurement carried out in this way is shown in the following tabulation in which, at the same time, there is indicated how many valencies each antitoxin fraction represents, on the basis of an assumed total of 200 for each unit.[16]

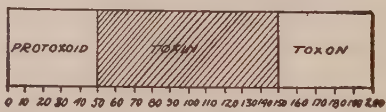
0	antix. unit representing	0	valency	$+ L_+ =$	85	free T units
.1	antix. unit representing	20	valencies	$+ L_+ =$	85	free T units
.25	antix. unit representing	50	valencies	$+ L_+ =$	60	free T units
.8	antix. unit representing	160	valencies	$+ L_+ =$	10	free T units
.9	antix. unit representing	180	valencies	$+ L_+ =$	3.5	free T units
1.0	antix. unit representing	200	valencies	$+ L_+ =$	1	free T unit

It is immediately evident in this table, as it would be evident in any other citation of similar measurements, that there is no regularity in the progress of neutralization; or, in other words, that addition of a definite fraction of antitoxin does not remove the arithmetically corresponding amount of toxic properties from the L_+ dose. The first 0.1 unit of antitoxin in this table has removed no free toxin whatever. The addition of the next 0.15 of an antitoxin unit, representing 30 valencies, has removed $^{25}/_{85}$ or $^{5}/_{17}$ of the total toxicity. Throughout the scale there is not the regular progression of neutralization, multiple by multiple, which would be expected if antitoxin could be titrated against a pure toxin. This, according to Ehrlich, is due to the presence of various toxoids which possess varying affinities for the antitoxin molecule. The first 0.1 of a unit added, in this case, does not diminish the toxicity of the mixture because it is bound by "protoxoids" which possess a higher affinity for antitoxin than the toxin itself. Next are bound the toxins themselves together with varying amounts of "syntoxoids" which possess the same affinity as toxin. Finally there are left the toxons which

[16] This measurement is taken from one cited by Ehrlich in *Deutsche med. Woch.*, No. 38, 1898, Vol. 24, and is taken literally except for the first value of 1/10 antitoxin unit, which is inserted to illustrate the formation of protoxoids.

possess a lesser affinity than toxins or toxoids, and therefore again have the discrepancy between the L_0 and L_+ dose. Ehrlich utilizes this method in order to determine the composition of the constituents of any given toxic filtrate and expresses the results in the so-called "toxin spectra."

The construction of these spectra and the principles underlying the measurements on which they are based are very clearly illustrated by Madsen,[17] from whose article the following type spectra are taken:[18]



Toxin Spectrum After Madsen, *Ann. de l'Inst. Past.*, Vol. 13, 1899, p. 57.

This figure represents the diphtheria filtrate composed of 50 valencies of protoxoid, 100 toxin and 50 toxon equivalents. The measurements in this case may be represented by the following tabulation:

L_0 + 1 antitox. unit = 200 valencies = 0 lethal dose
L_0 + .75 antitox. unit = 150 valencies = 0 lethal dose
L_0 + .25 antitox. unit = 50 valencies = 100 lethal doses
L_0 + 0 antitox. unit = 0 valency = 100 lethal doses

The following diagram, also from Madsen, represents the same poison after it had deteriorated to ½ its toxic power. L_0, therefore would contain only 50 toxic doses.

After Madsen, *Ibid.*, p. 578.

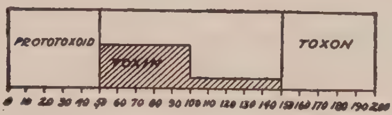
The measurements corresponding to this table are as follows:

L_0 + 1. antitox. unit = 200 valencies = 0 lethal dose
L_0 + .75 antitox. unit = 150 valencies = 0 lethal dose
L_0 + .745 antitox. unit = 149 valencies = 0 lethal dose
L_0 + .74 antitox. unit = 148 valencies = 1 lethal dose
etc., until
L_0 + .25 antitox. unit = 50 valencies = 50 lethal doses
L_0 + 0 antitox. unit = 0 valency = 50 lethal doses

[17] Madsen. *Ann. Past.*, Vol. 13, 1899, p. 576.

[18] We have chosen to illustrate the principles of the toxin spectrum from the article of Madsen rather than from Ehrlich's original work, because the former presents this difficult phase of the subject in a hypothetical toxin of extremely simple structure. Some of Ehrlich's spectra constructed from actual measurements may be found in *Deutsche med. Woch.*, No. 38, 1898.

The following spectrum, the third in Madsen's article, represents the same toxin described in the preceding spectrum but at a later period, at which considerable further deterioration had taken place. The L_0 dose now contained but 30 M L D or, in other words, the amount of toxin contained in the L_0 dose was sufficient to kill 30 guinea pigs only.



AFTER MADSEN, *Ibid.*, p. 579.

Madsen's description of the method in which this spectrum is constructed is the following:

$L_0 + {}^{200}/_{200}$	of an antitoxin unit kills	0 guinea pig
$L_0 + {}^{150}/_{200}$	of an antitoxin unit kills	0 guinea pig
. . . . .	. . . . .	. . . . .
$L_0 + {}^{141}/_{200}$	of an antitoxin unit kills	0 guinea pig
$L_0 + {}^{140}/_{200}$	of an antitoxin unit kills	1 guinea pig
$L_0 + {}^{130}/_{200}$	of an antitoxin unit kills	2 guinea pigs
$L_0 + {}^{120}/_{200}$	of an antitoxin unit kills	3 guinea pigs
$L_0 + {}^{100}/_{200}$	of an antitoxin unit kills	5 guinea pigs
$L_0 + {}^{99}/_{200}$	of an antitoxin unit kills	5 guinea pigs
$L_0 + {}^{98}/_{200}$	of an antitoxin unit kills	6 guinea pigs
$L_0 + {}^{97}/_{200}$	of an antitoxin unit kills	6 guinea pigs
$L_0 + {}^{96}/_{200}$	of an antitoxin unit kills	7 guinea pigs
$L_0 + {}^{90}/_{200}$	of an antitoxin unit kills	10 guinea pigs
$L_0 + {}^{50}/_{200}$	of an antitoxin unit kills	30 guinea pigs
$L_0 + {}^{1}/_{200}$	of an antitoxin unit kills	30 guinea pigs

The amount of toxon has remained the same in spite of deterioration. As less and less antitoxin is added, between the values of ${}^{150}/_{200}$ and ${}^{100}/_{200}$ of an antitoxin unit, there are now liberated only 5 fatal doses of the toxin. It is in this zone that deterioration has taken place, since, in the preceding spectrum, the difference between the addition of ${}^{150}/_{200}$ and ${}^{100}/_{200}$ of an antitoxin unit represented 25 fatal doses for guinea pigs. When in this last spectrum the amount of antitoxin is gradually reduced from 100 valencies to 50 valencies 25 fatal doses are liberated, a quantity corresponding to the similar zone in the preceding spectrum. Thus in this particular zone of the spectrum no change has taken place. The same is true of the protoxoid zone.

It is unnecessary to cite a larger number of such measurements in this place, since the ones given sufficiently illustrate the methods and the conclusions drawn from them. As a result of such experiments Ehrlich concludes:

I. That the diphtheria bacillus produces primarily two kinds of substances (a) toxin, (b) toxon, both of which bind the antibody.

II. The toxins (and perhaps also the toxons) may deteriorate and be modified into secondary substances (toxoids) which may be distinguished by their different degrees of affinity for antitoxin.

III. This classification does not exhaust all possible complications, since each subdivision of toxin consists apparently of equal parts of two different modifications which are similar to each other in their relation to antitoxin but differ in varying resistance to influences of deterioration. A more complete analysis of these conditions may be found, together with a series of illustrative spectra, in Ehrlich's article in the *Deutsche med. Wochenschr.,* Sept., 1898, which has been quoted above.

The complex deductions arrived at by Ehrlich are largely dependent, as we have seen, upon strict adherence to the analogy between the toxin-antitoxin reactions and those occurring between strong acids and strong bases. In such cases there is a complete reaction, in which chemical change ceases only when there has been a complete neutralization of one by the other. If, for instance, we mix molecular equivalent amounts of H_2SO_4 and NaOH, an apparently complete change into Na_2SO and H_2O occurs:

$$H_2SO_4 + 2\ NaOH = Na_2SO_4 + 2\ H_2O$$

The reverse process does not seem to take place, and if traces of uncombined H_2SO_4 and NaOH are present, as may be theoretically assumed, they are so slight in amount that they are not demonstrable. There are, however, many chemical reactions in which the process is not a complete one, in that the chemical change does not proceed until the reagents are completely used up. Reaction in these cases ceases when an equilibrium is reached at which there are present definite amounts of the reaction products and of the original substances at the same time.[19]

This occurs when a weak acid is added to a weak base. In such cases the reaction is incomplete and reversible and, together with the neutralization products, both free acid and free base may be present. The conditions are best explained by citing an example of a reversible reaction which is commonly given in text-books of physical chemistry, namely, the reaction between ethyl-alcohol and acetic acid. (Our citation is taken from Philip's "Physical Chemistry," London, Arnold, 1910): "When one gram mol. of ethyl alcohol is added to one gram mol. of acetic acid, a reaction takes place which results in the formation of ethyl acetate and water; the reaction, however, is incomplete and stops at an equilibrium point at which the reaction mixture contains ⅓ gram mol. alcohol, ⅓ gram mol. acid, ⅔ gram mol. ethyl acetate, and ⅔ gram mol. water. If, on the other hand,

[19] See Cohn. "Vorträge f. Ärtze über Physik. Chem.," Engelman, Leipzig, 1901.

1 gram mol. of ethyl acetate is mixed with 1 gram mol. of water, a reaction sets in which results in the formation of ethyl alcohol and acetic acid. This change likewise stops in equilibrium at a point at which the composition of the reaction mixture is the same as that already stated." The reaction is thus reversible and may be written:

$$C_2H_5OH + CH_3COOH \rightleftarrows CH_3COOC_2H_5 + H_2O$$

Another example somewhat simpler and more easily brought into analogy with the toxin-antitoxin reaction is that of the dissociation of phosphorus pentachlorid into phosphorus trichlorid and chlorin (see Alexander Smith, "General Chemistry," Century Company, N. Y., 1911, p. 181).

Here the reaction takes place:

$$PCl_5 \rightleftarrows PCl_3 + Cl_2$$

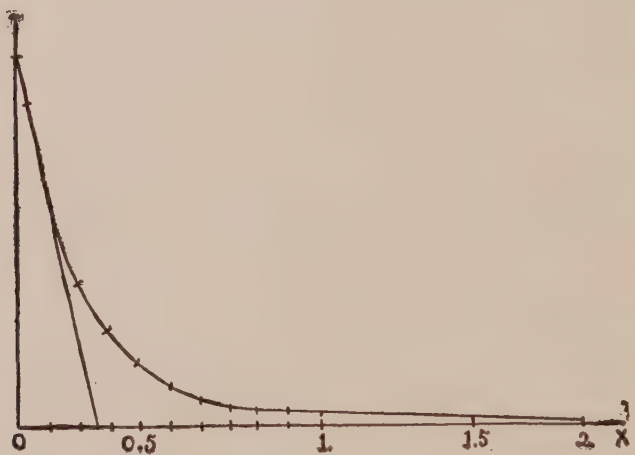
Chemical equilibrium is reached when the reaction speed is the same in both directions, and there will be present, at equilibrium, PCl_3, Cl_2, and PCl_5. Now the "Law of Mass Action" (Guldberg & Waage) states that reaction goes on at a velocity proportionate to the concentration of the reacting molecules. It is plain, therefore, that at the point at which the reaction takes place with equal velocities in both directions, that is, at the equilibrium point, a very definite relation of molecular concentrations must obtain, and this relation can be expressed as a formula. For the example given above this may be written as follows:

$$\frac{\text{Conc. } PCl_3 \times \text{Conc. Cl}}{\text{Conc. } PCl_5} = K \text{ (constant)}$$

This formula is expressed in words by Alexander Smith as follows: "If we change the amount of the pentachlorid placed in the vessel, or if we use amounts of chlorin and trichlorid which are not equivalent, the numerical value at equilibrium of each concentration will, of course, be different, but the product of the concentrations of trichlorid and chlorin, divided by the concentration of the pentachlorid, will always give the same numerical value for (K), the constant, at the same temperature."

Now to return to the application of these facts to the neutralization of toxin by antitoxin, if the reaction is one analogous to that of a strong acid and alkali, as cited above in the case of H_2SO_4 and NaOH, we would expect a complete neutralization of one by the other, multiple for multiple, and the explanation of Ehrlich based on the assumption of different toxin constituents, of varying affinities, and different pharmacological effects, is the only one which will account for the experimental results. Assuming, however, that the

reaction is one analogous to that taking place between a weak acid and a weak base—such as boric acid and ammonia—we have an entirely different state of affairs. For here the reaction goes on to a point of equilibrium, and in mixtures containing equivalent amounts of the weak acid and the base there will be present the reaction products and also small amounts of unbound free acid and free base. And according to the law of "Mass Action," the quantities of free acid and base present will depend entirely on the masses of the reagents put together. Thus, for each particular mixture of the two, different quantities of the original substances will be found uncom-



Curve Representing the Neutralization of Tetanolysin by Different Quantities of Antitoxin.

Taken from Arrhenius, "Immunochemistry," Macmillan, 1907, p. 175.

bined, and, furthermore, the gradual addition of one to the other will not have a neutralizing value in exact proportion to the amount added. Were the toxin-antitoxin reaction analogous to such chemical systems, then we could assume that every mixture of the two substances, whatever the relative amounts, would contain not only the united toxin-antitoxin molecule, but also varying amounts of dissociated free toxin and free antitoxin, the quantities of each depending, according to the law of mass action, upon the molecular concentrations obtaining in the individual mixture. This, indeed, is the conception of toxin-antitoxin union formulated by Arrhenius and Madsen.

Arrhenius and Madsen,[20] bearing in mind these conditions, made comparative studies of the neutralization of tetanolysin by its

[20] Arrhenius and Madsen. *Zeitschr. f. physik. Chem.*, 44, 1903, and *Festschrift Kopenhagen Serum Instit.*, 1902. Arrhenius. "Immunochemistry," Macmillan, N. Y., 1907.

antilysin on the one hand, and that of ammonia by boric acid on the other. Ammonia, like most bases, is a hemolytic agent, while boric acid, unlike stronger acids, has no hemolyzing properties. For this reason, in mixtures of the two, the toxicity is proportional to the concentration of free ammonia (though, as Arrhenius states, "a correction must be made for the lowering action of the ammonium salt, as indicated by experiments on this action"). Because the reaction between boric acid and ammonia is reversible, that is, the salt formed is dissociated by the hydrolytic effect of the water, there is always present a slight amount of free ammonia even if the largest possible quantities (to saturation) of boric acid are added. (See Arrhenius, "Immunochem.," p. 174.) The curve of toxicity indeed descends as more boric acid is added, but never reaches 0.

By a modification of the formula expressing the law of Mass Action, Arrhenius and Madsen could calculate the amount of free ammonia present in a series of mixtures in which increasing quantities of boric acid were added to a constant quantity of ammonia, and the values so obtained corresponded with much accuracy to those resulting from measurements of toxicity upon red blood cells. The following table taken from Arrhenius and Madsen illustrates this:

TOXICITY (Q) OF 0.1 N. NH_3 (1 EQUIVALENT) WITHIN EQUIVALENTS OF BORIC ACID. (Taken from Arrhenius, *loc. cit.* p. 176.)

n = Equivalents of boric acid added	Quantity of free ammonia—i. e., toxicity—observed	q = Ammonia toxicity calculated from formula	Δq obs.
0	100	(100)	
0.17	85	79	15
0.33	69	64	16
0.67	43	42	26:2 = 13
1	25	27	18:2 = 9
1.33	20	18	5:2 } 2.5
1.67	13	13	7:2 } 2.5
2	10	10	3:2 } 2.5

Here, in the last column, there is indicated the proportion of toxicity which is neutralized by the successive addition of ⅙ of an equivalent of boric acid. The first additions lower it to a degree proportionate to the amount of acid added; the next additions neutralize it to a much slighter degree, and, as further additions are made, each successive one possesses progressively less relative neutralizing power than the preceding.

This, it is plain, is closely analogous to the phenomena observed by Ehrlich in his "Partial Absorption" method, and Arrhenius concludes that the two phenomena, toxin-antitoxin and boric acid-ammonia neutralization, are closely analogous. His point of view is

further strengthened by his experiments with tetanolysin and its specific antibody, in which he constructed a curve similar to that given for boric acid, derived a formula and found that the observed and the calculated values closely coincided for various mixtures of the two. He claims, in consequence, that the phenomena observed by Ehrlich should not be interpreted as due to "partial toxins"—toxoids or toxons, but dependent rather upon the presence of varying quantities of free toxin dissociated from union with antitoxin because of the reversibility of the union.

The opinions of Arrhenius and Madsen are not generally accepted. It is in the first place doubtful whether substances like toxin and antitoxin, which, as far as we know their chemical nature at all, belong to the class of substances spoken of as colloids, can be regarded as subject to the laws of Mass Action in their reactions.

Nernst [21] has criticized Arrhenius' deductions chiefly on the basis of their assumption of the reversibility of the union of toxin and antitoxin, since reversible reactions between colloids, though not at all inconceivable, have so far not been definitely shown. Furthermore, as Nernst states, if complete reversibility of such reactions were possible it would be hard to understand how antitoxin can protect the animal against the actions of toxin.

These objections to the dissociation ideas of Arrhenius and Madsen do not, in our opinion, hold good any longer. Recent work upon the immunization of animals with partially and completely neutralized toxin-antitoxin mixtures, the work upon human immunization in diphtheria with similar preparations, and the increasing knowledge we are obtaining about the manner in which proteins may unite with other substances at different hydrogen ion concentrations, seems to prove that in regard to dissociation, proteins may follow laws not fundamentally different from those prevailing with other substances. Therefore, to refute the opinions of Arrhenius and Madsen entirely on the objection that dissociation does not take place with substances like toxin and antitoxin, is neither justified nor sound. This matter is referred to again below in connection with the analogy to enzyme reactions.

Another point of view concerning the toxin-antitoxin union which has been gaining ground especially through the work of Landsteiner and his pupils, is that of Bordet.[22] Bordet expresses his views in the following way:

I. When one mixes with a certain quantity of toxin an amount of antitoxin which is insufficient to produce a complete neutralization, the molecules of antitoxin are not taken up by a definite fraction of the toxin molecules, satisfying the affinities of these entirely

[21] Cited from Landsteiner in "Kolle u. Wassermann Handbuch," 2d Ed., Vol. 5.

[22] Bordet. *Ann. de l'Inst. Past.,* Vol. 17, 1903.

while other units remain intact; on the contrary, the antitoxin molecules distribute themselves equally upon all the toxin molecules present, and these are therefore, all of them, partially saturated, and lose proportionately a part of their initial toxicity. One could say that there is an attenuation of the toxin since there is a formation of a less poisonous complex.

II. The symptoms of poisoning produced by such a complex injected into animals or placed in contact with sensitive cells cannot be identical with those which would be produced by a fully saturated mixture of toxin and antitoxin, or by intact toxin.

III. Between these two extremes, free toxin and entirely neutralized toxin, one can imagine many transitions, progressive stages of attenuation. Every time that one mixes toxin and antitoxin in the same way one attains the same degree of attenuation.

Briefly put, this means that Bordet estimates toxin-antitoxin combinations of different degrees of toxicity as representing different stages in the completeness of the saturation of the individual toxin units. When 10 parts of toxin are added to 1 part of antitoxin, the result, according to him, would not be such that 1 part is neutralized by 1 part of antitoxin, leaving 9 parts of toxin free. He assumes rather that each unit of toxin is attenuated by the absorption of 0.1 of a part of antitoxin. He compares this process to the action of iodin upon starch. Starch can absorb variable quantities of iodin and, according to the amount taken up, is colored slightly or deeply blue. This mode of action is common to most staining processes. The substance that is stained fixes varying quantities of coloring matter and the coloring matter does not limit itself to a definite fraction of the substance stained but distributes itself equally to the material, coloring it slightly or deeply, in its entirety, according to the relative amount of color added. We will see later that there are many reasons for regarding other antigen-antibody combinations as following similar laws of proportion.

Bordet and others speak of this point of view as the "Adsorption Theory," and Biltz, in studying this point of view by physical methods, comes to the conclusion that the observed figures of the quantitative relations between toxin and antitoxin in the process of neutralization are fairly consistent with the values to be expected if the process were actually an absorption phenomenon.

Bordet [23] supports this point of view not only by test tube experiments, but also by general observations in animals. He cites, for instance, the observation that it takes more tetanus toxin to kill a guinea pig than a mouse, but if one takes into consideration the difference in weight between the two animals, it is found that the guinea pig is in reality more sensitive. Now, if a mixture of toxin and antitoxin is made of such strength that a mouse can still sur-

[23] Bordet, "Traité de l'Immunité," Masson, Paris, 1920, p. 530.

vive it, the same mixture may kill a guinea pig. Such an effect cannot be explained, as Bordet rightly argues, by traces of free toxin, but might well be explained by the fact that the toxin was sufficiently neutralized in its individual units to have no effect on the mouse, but not sufficiently so to permit poisoning of the guinea pig. According to Bordet, then, the quantitative factor, as well as the qualitative, that is, the degree of neutralization in the condition of each particular toxin unit must be considered in all experiments in which toxin and antitoxin are combined.

Bordet's conception of antigen-antibody union in this case can be brought out by other antibody reactions rather more easily than with toxin-antitoxin mixtures, and it is relatively easy to prove that cellular antigens, such as red blood cells and bacteria, can be sensitized with varying degrees of intensity, unit by unit, according to the concentration of the antibody solution with which they are brought in contact. Moreover, a very striking example of this was recently demonstrated in our laboratory in experiments on pneumococcus agglutination by J. T. Parker. When pneumococcus suspensions were brought in contact with antiserum and immediately shaken for a short time, agglutination occurred promptly. When the mixture was left unshaken, agglutination proceeded much more slowly. When shaking was delayed for three or four minutes after the first contact, the hastening effect of the shaking was no longer apparent. These observations seemed to us most easily explained as due to the immediate distribution of the agglutinating antibodies to the entire mass of the suspension in the cases where shaking was practiced.

One of the most important arguments in favor of Bordet's ideas as against the conception of Arrhenius and Madsen, and the establishment of a definite equilibrium, are the various experiments in which the reversibility of antigen-antibody reactions has been shown to be a very slight one only, and that, although an equilibrium is reached between the two, it is not affected by dilution and time factors in the same way in which the ordinary mass action phenomena take place. For instance, Madsen, while he has noticed that dilution can increase the toxicity of mixtures of toxin and antitoxin, has shown that this is only true of freshly mixed, but not of old preparations. Similarly, Madsen and Walbum have shown that diphtheria toxin and antitoxin diffuse into gelatin with different speeds; but that the difference in diffusion of mixtures is only noticed when the experiment is done with fresh toxin-antitoxin preparations. If the mixtures have stood for some time, the neutralized solutions no longer dissociate.

Again, the long train of analogies between antigen and antibody reactions in the case of precipitins, for instance, the necessity for proportionality of reacting substances, zone reactions, etc., which are

discussed in another part of the book (see chapter on Precipitins) supports the idea that these unions take place more like adsorption phenomena than like the chemical reaction between inorganic substances. While all this is true, we must nevertheless, remember that in view of the more recent advances in our knowledge of colloidal reactions, such as those being made by Loeb at the present time, may in the end show that, fundamentally, these reactions are not as different from each other as they now appear.[24]

A curious occurrence which seems to bring the toxin-antitoxin reactions close to colloidal reactions in general is that which is known as the "Danysz [25] Effect" or as the "Bordet [26]-Danysz Phenomenon." Danysz discovered that when ricin or diphtheria toxin were brought into contact with their homologous antibodies the degree of neutralization depended upon the manner in which the two were put together. When the toxin was added to the antitoxin in two fractions, a considerable time being allowed to elapse between the additions, the final mixture was much more toxic than when the total amount was added at once. In other words, although both mixtures contained exactly the same quantities of the two reacting substances, nevertheless the amount of toxin left free varied in the two cases, according to the speed with which they had been put together. This was confirmed in 1904 by von Dungern [27] for diphtheria toxin, and Craw [28] was able to observe it in the case of megatheriolysin and its antilysins.

A good deal of light may be hoped for in regard to the nature of antigen-antibody unions from investigations upon the nature of enzyme reactions. It was largely due to the researches of Duclaux,[29] Bayliss,[30] and their followers that the combination of an enzyme with a substrate was looked upon as taking place by adsorption.

[24] For a more complete discussion of this problem see Bordet, *loc. cit.*

[25] Danysz. *Ann. de l'Inst. Past.*, Vol. 16, 1902.

[26] Bordet. *Ann. de l'Inst. Past.*, Vol. 17, 1903.

[27] Von Dungern interpreted this in the sense of Ehrlich, by assuming it to be due to what he calls "epitoxonoids." This epitoxonoid he assumes to be a constituent of toxic broth, which has still less affinity for antitoxin than the toxon. It can combine with diphtheria antitoxin, but not until all the true toxin is bound. However, when it is once united with diphtheria antitoxin it is not very easily displaced from the union, especially when a considerable time has elapsed since the union. Therefore, he thinks, when the toxin is added to the antitoxin in two fractions, this epitoxonoid is bound and keeps the toxin, which is added later, out of combination. Whereas if the toxic broth is added as a whole, it is the epitoxonoid which is left unbound. This explanation of von Dungern's may be looked upon as an ingenious refinement of the reasoning introduced by Ehrlich into this field. Von Dungern. *Deutsche med. Woch.*, 30, 1904.

[28] Craw. *Jour. Hyg.*, Vol. 7, 1907.

[29] Duclaus. "Chemie Biol.," Paris, 1883.

[30] Bayliss. *Proc. Royal Soc. London*, Series B, 84, 1911, 90. Bayliss. "The Nature of Enzyme Action, Monographs of Biochemistry," London, 1914.

Northrop [31] has recently studied the reaction of trypsin and pepsin with its substrate in detail, and has, in our opinion, thrown considerable light upon the processes. In his pepsin studies he found that the apparent divergence of pepsin action from the results predicted from the law of mass action, can be quantitatively explained by the fact that the enzyme in solution combines with some of the substances [32] produced by the digestion, and maintains an equilibrium with this substance which obeys the ordinary laws of mass action. He bases his assumption that the pepsin combines with such substances as peptone (using the word "peptone" broadly, as signifying substances in solution with which the pepsin combines but does not hydrolyze) upon the experiments of Pekelharing and Ringer [33] who found that while pure pepsin solutions showed no iso-electric point when tested between two electrodes, the direction of migration of the pepsin was changed upon the addition of peptone at a Ph of 3, which is the iso-electric point of the added peptone. By such a conception Northrop explains the experimental fact that the rate of digestion of proteins by pepsin is not proportional to the total concentration of the pepsin. As the pepsin digests the protein, peptone-like substances are formed with which pepsin goes into combination and with which it then maintains an equilibrium following mass action laws. Since it is only the uncombined or dissociated pepsin which affects the further hydrolysis, the curves experimentally obtained may be readily explained. In adding increasing amounts of peptone to pepsin solution, as a result of this manner of combination, the first amounts added inactivate more pepsin than the later additions. We need hardly call attention to the fact that, as Northrop points out, this is in very striking analogy to the manner in which antitoxin and toxin react in Ehrlich's experiments.

In similar studies on trypsin action, Northrop confirms his pepsin studies in general. Here the process in its relative quantitative relations goes on as follows: At the beginning of the experiment, the trypsin solution contains undigested protein, and as the trypsin acts upon this, trypsin-inhibiting substances are formed. These will naturally increase during the experiment, and the observed quantitative results will consist in, first, a reversible inactivation of the trypsin due to the presence of the inhibiting substance, and, secondly, an irreversible destruction of free trypsin. The curve of trypsin activity resulting from this is one in which there is, at first, a very rapid drop of activity, which gradually becomes slower. In studying the effect of inhibiting solutions on the rate of digestion by trypsin,

[31] Northrop, J. H. *Jour. Gen. Physiol.*, 2, 1920, 471, and 4, 1922, 227, 245, 261.

[32] These substances are not, as once supposed, the amino-acids.

[33] Pekelharing and Ringer. *Zeit. f. Phys. Chem.*, 75, 1911, 282.

Northrop made another very interesting observation which is in very close analogy to the Dansyz phenomenon. When the inhibiting solution and trypsin were mixed, the act of mixing and the time of standing had no effect at ordinary temperatures. If the experiment, however, was done at 38°, the results were different. The solution in which the inhibitor (antitoxin) was added at the beginning, was much more active at the end of the experiment than one in which the inhibitor was added in fractions. In commenting upon this, Northrop states that the main discrepancy in the analogy is the fact that in the trypsin experiment the result is more marked if a relatively small quantity of trypsin is added in the beginning of the experiment, whereas, in the Dansyz it is necessary to add an excess. This he believes is due to the fact that in the trypsin experiment (toxin) the trypsin is changed during the experiment, while in the Dansyz phenomenon it is probably the free antitoxin (inhibitor) which is altered.

These experiments of Northrop do not, of course, solve the question of antigen-antibody unions, but they do serve to bring the analogy of toxin-antitoxin relations much closer to laws governing the union of enzyme with substrate. Moreover, they show that enzyme is actually used up in its reactions, just as toxin is used up in its reactions with antitoxin, and that equilibrium following the laws of mass action may be a definite factor in the quantitative relations governing the reactions. It is not at all impossible that the general laws which govern reactions between trypsin and its inhibiting substances are similar to those which govern the toxin-antitoxin reaction. Moreover, while it is a dangerous subject upon which to theorize, it is yet not utterly impossible that the toxins are closely analogous to enzymes, and that they produce in their action upon cells, products of injury which, passing into the circulation, become the specific inhibitors of the toxin or the antitoxin. We do not wish to dignify this with the label of a theory, but in subjects as vague as the origin and biological meaning of antitoxins, we must grasp at every straw that may suggest experimental paths for enlightenment.

THE SIDE-CHAIN THEORY

Mechanism of Antibody Formation

The discovery of antitoxins in the blood serum of toxin-immune animals by Behring and his collaborators furnished a point of new departure for the investigation of the phenomena of immunity, and Ehrlich's work upon the nature of the reaction between toxin and antitoxin, both in vitro and in the animal body, firmly established that the protective effect of the latter was one of direct neutraliza-

tion, and not, as at first supposed, one of toxin destruction or of indirect influence through the mediation of the body cell. As we have seen, moreover, it was quickly noted that these reactions were strictly specific in that an antitoxin produced with any one of the known toxins reacted solely with this one to the exclusion of all others. All these facts were of the utmost practical importance and gave hope of ultimate extensive therapeutic application, a hope which has, in part, been realized.

The physiological mechanism by which these phenomena were brought about, however, was, and is, to a great extent still, a mystery, and a most extensive and painstaking series of researches has occupied itself with its elucidation.

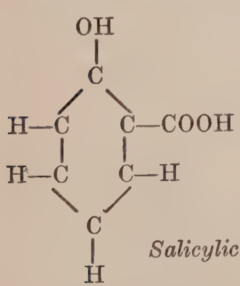
When we consider the invariable production of a specific antitoxin in response to the treatment of an animal with a toxin it is but natural that Buchner and others should have at first assumed that the antitoxin is, in each case, a product obtained by the action of the body tissues from the toxin itself. While difficult to refute at a time when little was known of the laws of antitoxin production and of quantitative relationships, such an assumption is entirely untenable in the light of more recent knowledge. We now know that such a simple conversion of toxin into antitoxin cannot explain the phenomenon because the amount of antitoxin incited in the immunized animal is out of all proportion great in comparison with the amount of toxin injected. Thus Knorr [34] has found that 100,000 units of antitoxin may be produced by the injection of the toxin equivalent of one unit. Moreover the discovery by Salomonsen and Madsen [35] that pilocarpin injections will increase the amount of antitoxin produced by an animal distinctly pointed to the likelihood of the participation of the general physiological activities of an immunized subject in the production of antibodies. Unquestionable proof of this was also brought by the experiments of Roux and Vaillard,[36] in which antitoxin production in immunized animals continued even after the entire volume of blood had been removed by repeated bleeding. This observation points distinctly to the direct secretion of antibodies by the tissue cell, in the nature of what has been termed by Roux [37] an "internal secretion." And it is this activity of the body cell in the production of antibodies which forms the fundamental premise from which the now classical "Side-Chain Theory" of Ehrlich takes its departure.

In order to approach this theory logically it will be of advantage to consider briefly the general subject of the assimilation of foodstuffs and other substances distributed by the circulation to the cells

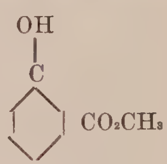
[34] Knorr. *Münch. med. Woch.,* 1898, pp. 321, 362.
[35] Salomonsen and Madsen. *Ann. de l'Inst. Past.,* Vol. 12, 1898.
[36] Roux and Vaillard. *Ann. de l'Inst. Past.,* Vol. 7, 1893.
[37] Roux. Ref. in *Semaine Medicale,* 1899.

of the animal body. For, as Ehrlich has expressed it, "The Reactions of Immunity, after all, represent only a repetition of the processes of normal metabolism, and their apparently wonderful adjustment to new conditions is only another phase of 'Uralter Protoplasma Weisheit.' "[38] It is impossible to conceive the nutrition of body cells without assuming that the assimilable nutritive substances come into physical and, eventually, chemical relationship with the protoplasm of the nourished cell. Considering the large variety of substances which may thus be brought into contact with cells in the course of normal and abnormal metabolism, the body cell, chemically considered as a complex of enormous molecules, must possess a correspondingly great variety of atom groups, by means of which it can unite with these substances to assimilate them and make them a part of its own protoplasm. In order to enter into similar relationship with toxins and other antigens, then, it is only logical to suppose that the cell, in the same way, unites chemically with the antigenic substance, and either assimilates it without sustaining harm, as in the case of non-poisonous complexes, or is injured in the process, as in the case of the poisons.

The living cell, from this point of view, is conceived as consisting of a central chemical nucleus, the "Leistungskern," more or less stable, in that the specialized tissue function is dependent upon it, and a manifold variety of "side chains," or atom groups by means of which it can enter into relationship with the nutritive and other materials carried to it by the body fluids. The latter term, "side chains," is taken from the nomenclature of chemistry, and, although the analogy is a loose one, it serves satisfactorily to elucidate Ehrlich's meaning. Thus we may conceive the "Leistungskern" as the central carbon ring of any compound of the Benzol series, as, for instance, in salicylic acid in which the hydrogen atoms, the hydroxyl,



Salicylic acid



Methyl salicylate

and the acid radicles represent "side chains." By means of the latter the compound can enter into relation with other substances, as, for instance, with CH_3 in the formation of methyl salicylate. Graphically, though this analogy formulates Ehrlich's fundamental

[38] Ehrlich. Introduction to "Gesammelten Arbeiten," Berlin, Hirschwald, 1904.

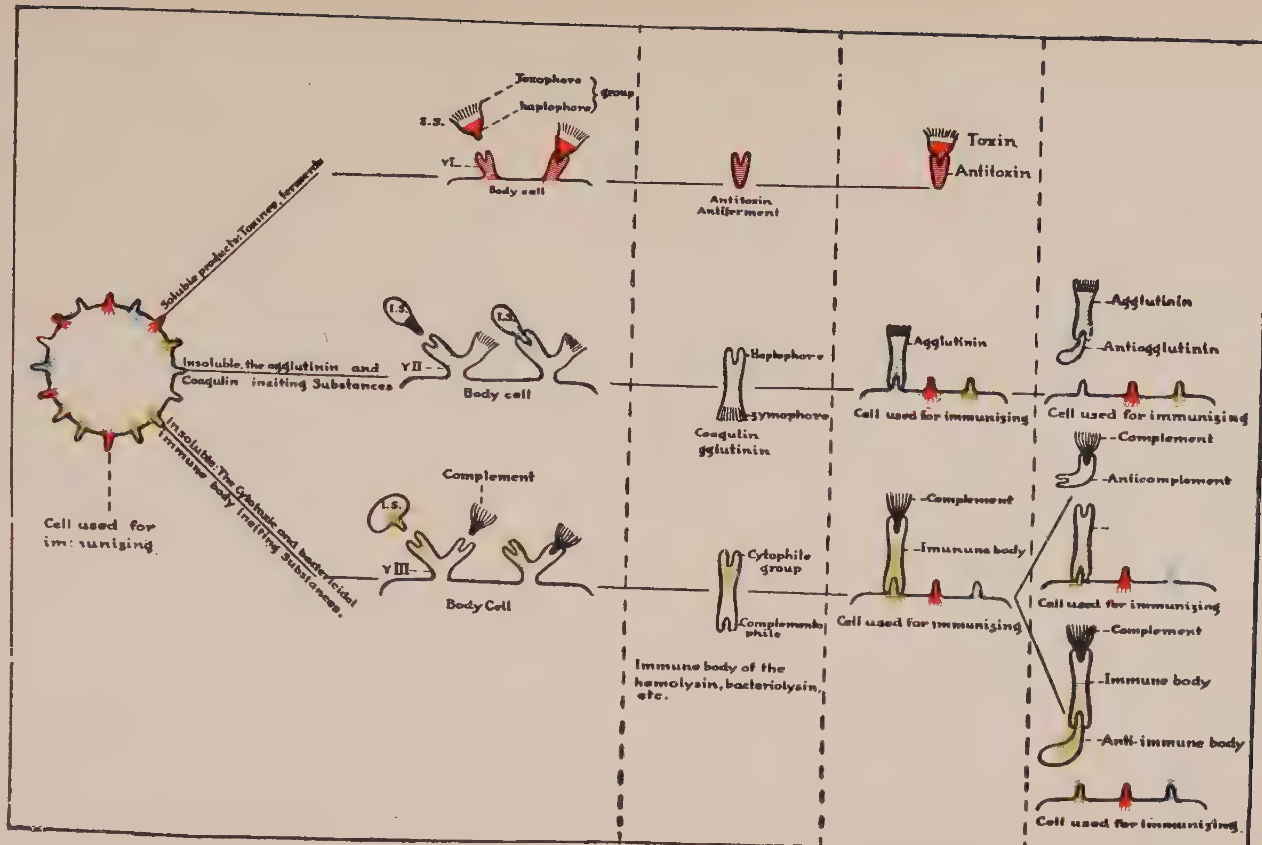
conception, it must not be taken as too literally representing the existing conditions, since, in actual metabolic interchange, there is an infinite variety of possible "side-chain" groups; for we are dealing with an enormous number of assimilable substances, most of them of chemically unknown constitution. The cell, therefore, is looked upon as an active chemical complex, retaining its own peculiar functional characteristics by reason of the "Leistungskern," but constantly getting rid of waste products and entering into new union with extraneous materials by virtue of its "side chains." These side chains, because of their "receiving" function, are spoken of by Ehrlich as cell "receptors."

That the chemical structure of certain bodies determines their ability to enter into relation with cell derivatives such as enzymes is, of course, a fact well established by experiment and explains the specific action of bacterial and other ferments upon certain substances to the exclusion of others. Thus Pasteur noted the fact that bacterial ferments could decompose dextrorotatory tartaric acid while they did not affect the levorotatory variety, and Emil Fischer [39] showed that only those carbohydrates possessing 6 and 9 carbon atoms were subject to fermentation by yeasts, and of these only the ones belonging to the "d" series, observations which, by demonstrating the relationship between these active agents of extracellular digestion, and the stereochemical configuration of the molecules acted upon, lend much support to the logic of Ehrlich's contentions.

Moreover, the recent experiments upon the growth of tissues in plasma outside of the animal body in which cartilage cells produce cartilage, kidney cells, etc., have shown that, given the same nutritive materials, the cells themselves must command a certain selective power in the choice of these materials, which can only depend upon a specific element in the structure of the cell receptors. As Fischer has expressed it for fermentation, the ferment must possess an atom group which fits into some group of the fermentable substance as a "key does into a lock," an analogy which is equally applicable to Ehrlich's conception of the relation of the "side chain" to a nutritive molecule.

Now the toxins and other antigens are, all of them, so far as we know, complex chemical substances, derivatives of animal and vegetable cells, and, for this reason, should have much in common with the materials available for nutrition. It is not strange, therefore, that, coming into contact with the cells of the body during the accidents of disease or other abnormal conditions, they should find receptors by means of which they can combine with the cell. Under the ordinary conditions of nutrition a suitable particle taken up by the cell in this way would be assimilated and the receptor either

[39] See Oppenheimer, "Die Fermente," Vol. 1.



The Structure of Cell-Receptors and Immune Bodies, According to Ehrlich's Conception.
(After Aschoff.)

freed for further use or regenerated for the further absorption of similar substances, by virtue of a mechanism delicately coördinated to the needs of cell-nutrition. In the case of the absorption of substances belonging to the class of antigens, however, foreign proteins difficult of assimilation, or of toxins even directly harmful, the receptors occupied by these substances are rendered useless to the cell, and, if the cell continues to live, must be regenerated. If the degree of poisoning or the amount of other antigen introduced has been extremely slight, this regeneration may possibly take place, as in the course of nutritive processes, without further disturbance. If, however, the amounts of antigen are greater than this, or are repeatedly thrust upon the cell, the process of regeneration may be not only sufficient to compensate for the loss of the eliminated receptors, but may follow the general law of overcompensation, formulated by Weigert, and receptors of the variety occupied by the antigen are produced in excessive number.

Here again Ehrlich has called analogy to his aid, and has taken his conception of "overcompensation" from the well-known phenomena of pathological anatomy where, for instance, in the restoration of cellular elements after injury, there is often an overproduction of granulation tissue, far beyond the needs of simple healing.

Thus the restitution of cell receptors, if sufficiently stimulated by large quantities or repeated administration of the antigen, far exceeds the quantity normal to the cell, and may proceed to such a degree that the cell, becoming as it were "top-heavy" with these elements, sloughs them off into the surrounding lymph and blood, where they circulate as free receptors. These free receptors then, having specific affinity and combining power for the antigen which incited their production, unite with subsequently introduced antigen in the blood stream, diverting it from the cells themselves, and, in the case of the variety of antigens spoken of as toxins, this union with the free receptors in the blood stream would serve to protect the cells from harm, exerting thereby an antitoxic action.

The antibodies appearing in the blood of immunized animals, therefore, represent atom complexes, normally parts of the body cells and concerned in the metabolic processes, but now produced in excess and extruded into the body fluids under the influence of the stimulation of immunization. The very substances, as Behring has put it, which make possible the poisoning of the cell by the toxins become protective when, detached from the cell, they circulate in the blood. Thus the theory, beside explaining the causes leading to antibody formation, offers a plausible reason for the relatively strict specificity observed in antibody-antigen reactions.

Formulated in direct connection with the investigations upon toxins and antitoxins, the side-chain theory has been extended by Ehrlich and his associates to all known phases of antibody-antigen

reactions. The differences in the nature and complexity of various antigens would naturally necessitate variation in the receptors capable of assimilating them, and these receptors, appearing subsequently in the blood as antibodies, must, of necessity, differ from each other. On this basis Ehrlich has conceived of three main varieties or "orders" of receptors or "haptines," as he calls them. Of these the simplest are those of the first order which attach to the *toxins,* and by over-regeneration appear in the blood stream as *antitoxins.* Those of the second order, adapted to the assimilation of more formidable protein molecules, are, of necessity, of greater structural complexity, appearing in immunized animals as the *agglutinins* and *precipitins,* while those of the third order, dependent upon the cooperation of alexin or complement, for proper functionation, appear as the *cytotoxins* or *lysins.* The detailed structure of these various haptines will be discussed in connection with other considerations dealing with their special reactions.

Limiting ourselves, for the present, to a broad consideration of the theory as a whole, it may be briefly recapitulated as follows: Toxins or other antigens, in order to exert any influence upon the animal body, must enter into chemical relationship with the cells. This they do by virtue of union with chemical units or atom groups of the cells, spoken of as "side chains." These side chains or receptors, thrown out of function by this union, and necessary for the metabolic processes of the cell, are regenerated, and under the influence of repetition of this process are produced in excess, to such a degree that they are eventually thrown off by the cells and enter the circulation as antibodies. Thus far the theory, comparing the union of antigen with cells to the processes of nutrition, is eminently logical and likely, necessitating the assumption of over-regeneration as the only criterion not directly amenable to experimental proof.

That the antigen can be bound by the body cells has been variously shown in a large number of investigations, some of which have been reviewed in our section on the action of bacterial poisons. We have there seen that Dönitz demonstrated the rapid disappearance of tetanus and diphtheria toxins from the circulation of susceptible animals, and that conversely Metchnikoff showed that the poison may persist unabsorbed and unchanged for weeks and months in the blood of such insusceptible animals as the turtle and the lizard, facts which furnish indirect evidence of the absorption of the toxins by the body cells. More direct evidence has, of course, been possible in the test tube experiments with hemolytic and other cell poisons where a directly specific combination between antigen and antibody has been easily demonstrable. Thus, in his earlier experiments with spider poison, Sachs was able to show that rabbit erythrocytes, which are sensitive to the poison, could absorb it out of solution, while dog and other corpuscles, which were insusceptible to the

poison, did not bind or absorb it. This can be easily demonstrated for many antigens and antibodies and may be accepted as a fact.

This point established, and repeatedly confirmed, and the origin of antitoxins from the cells of the body having been rendered likely by the experiments of Salomonsen and Madsen, and by those of Roux and Vaillard just cited, it would follow, by the theory of Ehrlich, that we should find the site of antibody production in the very cells which possessed specific affinity (receptors) for the antigen. This question has been variously investigated, chiefly in the case of the toxins and antitoxins, since this phase of the subject is most easily amenable to experiment. It will be remembered also that Wassermann and Takaki discovered that emulsions of the tissue of the central nervous system of rabbits and guinea pigs, shown by Meyer and Ransom and others to be the special points of attack for tetanus toxin, possessed the power of neutralizing this poison *in vitro,* while emulsions of spleen, kidney and other organs had no such effect. They assumed from this that the poison was fixed by cell receptors, antecedents of antitoxin in the sense of Ehrlich. Kempner [40] made similar observations with botulinus toxin and further confirmation has been derived from experiments like those of Blumenthal,[41] who found that the toxin was neutralized by the brain tissue of susceptible animals but showed conversely that the brain substance of the chicken, an animal but slightly susceptible to tetanus, possessed little or no neutralizing power. Similar results were obtained by Metchnikoff in the cases just cited.

The great importance of these experiments lies not only in showing that body cells may absorb the toxins, but that there is direct relationship between the susceptibility of tissue and the toxin-binding properties. Furthermore the facts demonstrated by Metchnikoff that no antitoxin was produced by those animals (turtle, lizard) in which the tissues had no power of fixing poison and are consequently insusceptible, furnish evidence in favor of Ehrlich's view.

It becomes of great importance, therefore, to determine whether in the case of the fixation of tetanus toxin by the brain cells the union between cell and toxin is a specific and chemical one comparable in every way to the union of toxin with antitoxin.

Metchnikoff, in spite of his results in the experiments just cited, objected to this interpretation on the ground that although the brain emulsion of a guinea pig neutralized tetanus toxin *in vitro,* the injection of the toxin into such an animal, subdurally, produced the disease. This can hardly be regarded as a valid argument against Wassermann's interpretation, since the very premises of the Ehrlich theory require that these neutralizing elements, when still attached

[40] Kempner and Pollak. *Deutsche med. Woch.,* 1897, No. 23, p. 505. Kempner and Shepilewsky. *Zeitschr. f. Hyg.,* Vol. 36, 1901, p. 1.

[41] Blumenthal. *Deutsche med. Woch.,* 1898, No. 12, p. 185.

to the living cell, as "sessile" receptors, are the cause of the poisoning, since they serve to "unlock" the cell to the entrance of the toxin. Similar objections on the part of Metchnikoff [42] were based on some of his own experiments, as well as on those of Courmont [43] and Doyen, in which it was found that the poison disappears but slowly (in 2 to 3 months) from the circulation of frogs, and the brain cells show hardly any toxin neutralization *in vitro,* whereas these animals can be rendered tetanic if they are warmed to 25° to 30° C. Further work, however, by these authors as well as by Morgenroth [44] has satisfactorily cleared up this difficulty. As a matter of fact, tetanus poison disappears more rapidly (that is, is bound by the cells more rapidly) from the circulation of frogs, if the frogs are warmed to 30° C. or more. Furthermore, if the toxin is injected into these animals, and they are kept at low temperatures, no disease results, but if they are then warmed up to the temperature stated, they gradually succumb to the disease. Morgenroth has shown that the apparently anomalous behavior of frogs in this respect is actually a question of temperature. At low temperatures the poison is bound, though with extreme slowness, but the toxophore group of the toxin does not functionate. When the animals are warmed, not only does the binding proceed more rapidly, but the toxophore group becomes active. He thus not only has answered Metchnikoff's objections to Ehrlich's theory on this ground, but has furnished an additional indirect confirmation of the dual constitution of toxin, that is, its constitution of a haptophore and a toxophore atom group, suggested by Ehrlich in his diphtheria-toxin analysis.

There is apparently, then, a strong absorption of tetanus toxin by the brain and nervous tissue of all animals which are susceptible to the poison, an absorption which amounts, as we have seen, to neutralization, the brain emulsion acting like antitoxin when mixed with the toxin before injection, as in Wassermann's and Takaki's experiments.

A serious objection has been brought, however, to the assumption that this binding can be identified in its nature with the similar binding of toxin by antitoxin, and a number of authors have claimed that the binding by the brain is not a binding by specific receptors, but an accidental property due to the presence of some fortuitous fixing substance in the central nervous system. Besredka [45] showed, for instance, that the brain of susceptible animals could bind much more toxin than it could actually neutralize, and that, if antitoxin was added to a brain emulsion previously saturated with the toxin, the toxin is removed from its combination with the brain cells and these

[42] Metchnikoff. *Ann. de l'Inst. Past.,* Vol. 12, 1898.

[43] Courmont and Doyen. *Arch. de Physiol.,* 1893. Courmont and Doyen. *Compt. rend. de la soc. de biol.,* 1893.

[44] Morgenroth. *Arch. internat. de Pharm.,* Vol. 7, 1900, pp. 265-272.

[45] Besredka. *Ann. de l'Inst. Past.,* Vol. 17, 1903, p. 138.

again regain their original absorbing property. These experiments would seem to point to a difference, especially in regard to affinity and firmness of union between the nature of the combination between toxin and brain emulsion on the one hand, and toxin and antitoxin on the other. This, of course, would prove a serious obstacle to the interpretation of the binding of toxin by susceptible cells in the sense of Ehrlich, as depending as it were upon union with specific receptors, or, as they might be termed, "sessile" antitoxin. Moreover, to strengthen such objections to this point of view, the work of Landsteiner and v. Eisler [46] has brought out the fact that extraction of brain tissue with ether materially reduces its toxin-binding powers by removing fatty or lipoidal substances, such as cholesterin and lecithin. And it has indeed been confirmed that lipoids can possess, in many instances, binding properties not only for toxins but for other forms of antibodies. On the basis that at least a part of the toxin absorption by brain emulsions depends upon such lipoidal fixation, the results of Besredka are readily explained, but were this the sole cause of toxin fixation by these tissues it would indeed be difficult to interpret the phenomenon, with Wassermann and Takaki, in support of Ehrlich's theory. For, without going into further refinements, the fact of the probable proteid, certainly not lipoidal, nature of the antitoxins, discussed in a previous section, would alone serve to distinguish the two modes of toxin fixation.

However, a number of facts have been ascertained which show that, although the lipoids play some part in the antitoxic action of brain cells, they do not by any means account for the entire process. In the first place it is found that the heating of brain emulsions almost completely removes their power to bind the toxin, while no such reduction of the fixative property follows the heating of lipoids like cholesterin or lecithin. The experiments of Marie and Tiffeneau [47] have done much to clear up the confusion regarding this point. They determined that the "lipoidal binding" constituted only about one-tenth of the total binding power of the brain emulsions, by showing in the first place that only one-tenth of the total was left after heating, and that all but one-tenth could be destroyed by subjecting the tissue to the action of proteolytic enzymes. It appears from this that a large part, at any rate, of the toxin fixation of the brain tissues is dependent upon substances of an albuminous nature, a smaller but definite part being dependent upon fixation by lipoids, a phenomenon entirely apart from the former in underlying principles. This would, it seems, both justify the original interpretation of Wassermann and still explain the apparently contradictory results of Besredka and others.

[46] Landsteiner and v. Eisler. *Centralbl. f. Bakt.*, Vol. 39, 1905.

[47] Marie and Tiffeneau. *Ann. de l'Inst. Past.*, Vol. 22, pp. 289 and 644, 1908.

CHAPTER VI

THE BACTERICIDAL PROPERTIES OF BLOOD SERUM, CYTOLYSIS, AND SENSITIZATION

In spite of the profound physiological alteration of the animal body which is implied by the acquisition of immunity against any particular infection, we have seen that no anatomical or histological changes in the organs and tissues accompany such alteration. The same is true of the difference between animals of different species, in which the most marked variation in resistance against any given infection is inexplicable on the basis of structural or microscopic characteristics in the organs. We have mentioned briefly the attempts that have been made to discover chemical and physical changes or differences to account for such conditions and have seen that the attention of investigators was soon attracted to the blood.

A possible relationship between the blood and the defence of the body against infection had been foreshadowed by observations made long before the days of bacteriological knowledge. As early as 1792, John Hunter, in his "Treatise on the Blood, Inflammation and Gunshot Wounds," had noted that the blood did not decompose as readily as other putrescible material, and a century later, during the period of great interest in the living nature of fermentation and putrefaction, Traube (1874) expressed the opinion that blood could destroy bacteria. Similar observations were made by Lister and by Grohman [1] but no experimental work aimed at this point was carried on until 1886, when the subject was taken up by Nuttall,[2] von Fodor,[3] and Flügge, and a little later by Buchner.[4] These authors, working with defibrinated blood, peptone blood, and blood serum, showed that such substances all exerted a definitely measurable destructive influence upon bacteria, and Nuttall, later confirmed by Buchner, further found that this bactericidal power was weakened on standing, and could be rapidly destroyed by heating to 60° C.

Their method of procedure consisted in the planting of controlled amounts of various bacteria in measured quantities of blood and, after several hours at 37° C., pouring plates and thus determining the numbers of surviving organisms. The fact of bactericidal power

[1] Grohman. Quoted from Adami, "Principles of Pathology," Vol. 1, p. 497.

[2] Nuttall. *Zeitschr. f. Hyg.*, 4, 1888.

[3] Von Fodor. *Deutsche med. Woch.*, 1887.

[4] Buchner. *Centralbl. f. Bakt.*, Vol. 5, 1889.

established, there was, of course, much early difference of opinion as to the mechanism responsible for the destruction of the bacteria, and a number of simple explanations were suggested which, though entirely refuted at the present time, still possess considerable interest in showing the stages of development through which the conceptions of the mechanism of immunity have progressed.

These early theories were formulated chiefly upon the underlying thought that the animal body was primarily passive in its relation to the invading micro-organisms, and that the disappearance of bacteria in the body fluids was due to the existence of a chemically or physically unfavorable environment which prevented their multiplication and therefore induced gradual mortality among them. Thus Billroth [5] believed that bacteria could thrive in the body only after a preceding putrefactive change had prepared a favorable pabulum. Others attempted to discover a relation between the degree of alkalinity of the blood serum and the destruction of bacteria. This argument was soon refuted by the experiments of Buchner, who showed conclusively that the bactericidal power of serum was not reduced by the neutralization of its natural alkalinity with weak acetic acid.

Another theory which has been kept alive until the present day by Baumgarten,[6] and in favor of which much has been written by Fischer, is the so-called "Osmotic" explanation. The basis of this conception is the observation that vegetable and other cells, which are in themselves delicate osmotic systems, undergo changes when they are placed into fluids of different osmotic tension.[7] Thus, of course, cells of all kinds may be destroyed by being placed in distilled water on the one hand, or in hypertonic salt solution on the other. The point of view of Baumgarten, as explained in a recent edition of his "Text-book of Bacteriology," is the following: The bacterial (or blood) cell, like all cells, is surrounded by a semi-permeable membrane. Under ordinary conditions, this membrane permits the passage of certain substances which must enter and leave the cell in the course of normal metabolism. When the bacteria are placed in a specific bacteriolytic serum there is a chemical union between the antibody and the cell membrane, and the latter is, in consequence, injured. The result of the injury is that now the cell becomes permeable for salts and other substances to which it was impermeable before, and there are consequent swelling and increased intracellular pressure. This, in turn, brings about the extrusion from the cell of proteins and other ordinarily non-diffusible substances, and destruction of the cell results. This explanation is practically an adaptation of the earlier more primitive osmotic theories to the facts subsequently discovered. It stands in direct contradiction to the prevailing opinion that the process of bacteriolysis and cytolysis in general is an enzymotic process, brought about by the injury of the cell by specific substances comparable to digestive ferments. Interesting though the suggestion of Baumgarten is, it can hardly

[5] Billroth. Quoted from Sauerbeck, "Die Krise in der Immunitätsforsch.," Klinkhardt, Leipzig, 1909.

[6] Baumgarten. "Lehrbuch der pathogenen Mikroorg.," Hirzel, Leipzig, 1911.

[7] See also Pfeiffer's "Pflanzen Physiologie."

receive more than casual attention given it for the sake of completeness, since careful experimental work by von Lingelsheim [8] has shown definitely that altered salt contents of serum do not exercise the effect upon bacteriolysis which we would be entitled to expect from Baumgarten's reasoning.

In explanation of the natural immunity possessed by many animals against various infections, Baumgarten has offered another explanation which, like the preceding, we may classify, in agreement with Sauerbeck,[9] with the "passive" theories. This theory, which he calls his "Assimilation Theory," assumes that the bacteria do not find suitable food material in the tissues and fluids of certain animals, and, since bacteria do not have to be killed to be eliminated, but may be checked merely by their inability to grow and multiply, they must soon succumb in surroundings in which they find no suitable foodstuffs. This point of view approaches somewhat the earlier exhaustion theory of Pasteur, which has been mentioned in another place.[10]

In contrast to these "Passive" theories of immunity are the now prevailing and well-founded opinions that the resistance of the animal body against bacterial invasion is not a mere fortuitous result of chemical and physical conditions encountered by the infectious agents, but is rather the result of the struggle against the invasion by active forces of the body cells and fluids. The part played by the cells had already been emphasized by Metchnikoff and his school when the discovery of the bactericidal power of the normal blood was made. The study of the antibacterial powers of the blood now introduced a new element which became the basis of the so-called "humoral" theories. In the prolonged controversies waged, with great astuteness and experimental skill, between the adherents of these two schools, most of the facts which we possess regarding immunity were discovered, and it is only within recent years that we have obtained information which has made possible a correlation between these two main paths of thought.

The humoral theory was conceived by Buchner, as the first important theoretical result of Nuttall's discovery. Buchner, as we have seen, confirmed the observations of Nuttall both as to the primary fact of the bactericidal power of the fresh normal blood and as to the unstable nature of this bactericidal property. He looked upon the antibacterial power as depending upon a constituent of the fresh blood plasma, which he named *"alexin"* (protective substance), and which he believed to be comparable to a proteolytic enzyme. The action of this alexin was conceived as potent against all bacteria equally, without showing specific selection of various species to any great extent. The analogy to ferment action was formulated by Buchner because of the heat sensitiveness and the instability of the bactericidal substance on standing; and he

[8] Von Lingelsheim. *Zeitschr. f. Hyg.*, Vol. 37, 1901.

[9] Sauerbeck. "Die Krise in der Immunitätsforschung," Klinkhardt, Leipzig, 1909.

[10] The influence of foodstuffs, temperature, and other environmental conditions upon natural immunity has been discussed in an earlier section.

suggested that this alexin might possibly be a product of the tissue or blood cells, possibly leukocytic in origin.

Buchner found that the action of the ferment-like alexin upon bacteria was most marked at the temperature of the body, and that it was capable of destroying bacteria in the subcutaneous tissues and the serous cavities of the animal body, without the aid or coöperation of cellular elements. He inferred that there was a direct relation between the potency of the alexin and resistance against infection.

The Pfeiffer Phenomenon.—The next great step in the understanding of the bactericidal processes was now made by Pfeiffer as a consequence of studies upon the nature of cholera immunity. Pfeiffer [11] found that the injection of cholera spirilla into the peritoneal cavity of a guinea pig which had recovered from a previous cholera infection was followed by a rapid destruction of the bacteria. If small quantities of exudate were taken out of the peritoneum at varying intervals after the injection, a granular change and swelling of the bacteria were noticed, followed, soon after, by complete dissolution and disappearance. Such animals would recover from doses of bacteria which, in control animals of the same weight, resulted in death. He further found that the phenomenon was specific, in that the dissolution of cholera organisms only occurred in the cholera-immune animals, other bacteria being unaffected. In other words, the guinea pig had acquired a specific antibacterial power, expressed by the process of "bacteriolysis," a property possessed to only a very slight extent by the peritoneal exudate of a normal animal. It was the next logical step to determine whether the bacteriolytic power could be transferred to the peritoneal cavity of a normal animal by injecting, together with the bacteria, a small amount of the serum of such an immune animal. This was indeed found to be the case and, although such immune serum, like normal serum, is deprived of its in vitro bactericidal power on heating, Pfeiffer found, in his intraperitoneal experiments, that heated serum is quite as effectual as fresh immune serum in transferring passive immunity to a normal guinea pig. We may summarize the important harvest of facts obtained from these experiments of Pfeiffer in the following statements:

1. Rapid dissolution of cholera spirilla takes place in the peritoneal cavity of a cholera-immune guinea pig. Similar lysis takes place not at all, or only to a slight extent, in the peritoneum of a normal pig. In consequence of the lysis the immune pig will survive the injection of quantities of bacteria which invariably kill normal animals of the same weight.

2. The protection obtained in this way is specific.

[11] Pfeiffer. *Zeitschr. f. Hyg.*, Vol. 18, 1894; also Vols. 19 and 20. Pfeiffer and Isaeff. *Deutsche med. Woch.*, No. 18, 1894.

3. The protection may be transferred from an immune to a normal guinea pig, by injecting a little immune serum together with the bacteria into the peritoneum of the normal animal. In a normal animal so treated lysis is in every way similar to that observed in the immune pig.

4. The transfer of the lytic power and consequent immunity can be brought about not only by means of fresh immune serum but by heated serum as well, although the latter has lost all its alexic power because of the heating.

Of the phases of this "Pfeiffer phenomenon" the one most difficult to understand, in the light of the knowledge of that time, was the transference of the lytic property with the heated serum. Pfeiffer very naturally took his experiments to signify that the actual destruction of bacteria in the animal body could take place entirely without the phagocytic participation of the body cells, a view in sharp contrast to that of the Metchnikoff school, and based upon his observation of the complete extracellular disintegration of the spirilla in the peritoneal exudate. He assumed, however, that there was an indirect participation on the part of the cells. The observation that heated serum, inactive outside the body, was efficient when introduced into the peritoneum, persuaded him that the coöperation of the living tissues was a necessary factor, and he assumed a possible activation by substances derived from the endothelial cells lining the peritoneal cavity. In the same way he explained his failure to observe actual bacterial dissolution in hang-drop preparations, even when fresh serum was used in the experiment.

It will be interesting to examine a protocol of an experiment such as those carried out in the performance of the Pfeiffer phenomenon in order to make the actual occurrences entirely clear. In such experiments the quantity of bacteria used must be chosen with some regard to the virulence and toxicity of the particular culture employed, since, as we shall see, protection of animals by bactericidal or bacteriolytic sera does not follow the law of multiple proportions as in the case of the protection against toxins by antitoxins. While the dose of bacteria chosen should be considerably above the minimal lethal dose for an animal of the weight used, it should nevertheless be remembered that the bactericidal serum does not possess antitoxic properties against the poisons liberated or produced as the bacteria undergo dissolution, and at best the protection by bacteriolysis is limited to a very definite maximum of bacteria, beyond which no further increase of serum quantity will avail. The following table will illustrate an experiment of this kind in which, in a series of guinea pigs, the bacteriolytic protective power (titre) is determined by comparative tests.[12]

[12] For extensive discussion of the technique of such tests see Boehme in *Kraus u. Levaditi Handbuch,* etc., Vol. 2, p. 366. The scheme of presenta-

PFEIFFER PHENOMENON

Weight of guinea pig	Dose of bacteria* cholera spirilla	Amount of inactivated immune serum	Result
(1) 215 gm.	2 mg.	0.1 c. c. in 1 c. c. salt solution.	Complete dissolution in less than 1 hour. Lives.
(2) 230 gm.	2 mg.	0.05 c. c.	About the same as first.
(3) 200 gm.	2 mg.	0.01 c. c.	Somewhat slower than in other two; a few unchanged spirilla after 1 hr. Final dissolution. Pig lives.
(4) 245 gm.	2 mg.	0.005 c. c.	Similar to (3) but complete dissolution in 2 hrs. Pig lives.
(5) 220 gm.	2 mg.	0.001 c. c.	After 30 min. the spirilla seem to have begun to multiply. Dies with innumerable active spirilla in peritoneum.
Normal control			
(6) 210 gm.	2 mg.	0.1 c. c. normal inactive rabbit serum.	Very slight lysis at the beginning. Soon rapid multiplication. Dies.

* The bacteria may be measured for such an experiment by standard loopfuls (1 loop being equal to 2 milligrams), or by volume in emulsion with salt solution.

Pfeiffer has established a system of standardization for the measurement of sera by this technique. He speaks of one immunity unit as the smallest amount of such a serum which is capable of causing complete dissolution of 2 milligrams of culture material[13] (of a standard culture) and saving the life of the animal. The unit of the serum in the preceding test would accordingly be 0.005 c. c., and the titre of the serum, expressed in Pfeiffer's language, would be 200 units to the cubic centimeter. Owing to the great variation in the virulence and toxicity of different strains of the same organism, and also because of the difficulties opposed to the visible dissolution of many bacteria, which may be killed by the serum without showing much evidence of solution, the practical application of Pfeiffer's standardization is not universally possible. In doing experiments by this technique, whatever their purpose may be, accurate adjustment of bacterial amounts and preliminary studies of virulence must be made in order that the tests may be of real value and, failing visible lysis, the death of the animals must be taken as the indicator

tion of our example is taken from that used by him. See also Pfeiffer, *Zeitschr. f. Hyg.*, Vol. 19, 1895, p. 77.

[13] The standard "loop" used in many laboratories for the rough measurement of quantities of bacteria from agar cultures takes up approximately 2 milligrams of the material.

of the titration. Comparisons of results obtained with two different cultures of the same species are consequently of value only when the minimal lethal dose of each and its toxicity have been studied before the final tests are made.

The cardinal points of Pfeiffer's phenomenon were rapidly confirmed, but his assumption that the process could take place only within the animal body was soon corrected by both Metchnikoff [14] and Bordet.[15] Both of these investigators succeeded in producing extracellular lysis of cholera spirilla in hang-drop preparations. The former produced the phenomenon by adding to the hang-drop preparations small quantities of extracts of leukocytes, and thus attempted to correlate Pfeiffer's observations with his own opinions regarding the importance of the leukocytes in bacterial destruction. The latter, however, subjected the phenomenon of bacteriolysis, both in vivo and in vitro, to a careful analysis and obtained results which definitely disproved the necessity of cellular intervention in this phenomenon, and furnished facts regarding the process which stand uncontradicted to the present day. Upon the basis of these our modern views of the mechanism of cytolysis in general are founded.

Mechanism of the Bacteriolytic and Hemolytic Process.—Bordet showed that the bacteriolytic properties of immune serum are indeed destroyed by heating to from 50° to 60° C. If, however, to such a heated immune serum there is added a small quantity of fresh normal serum, the bacteriolytic power is restored with undiminished vigor. He recognized in consequence that there were two distinct serum elements necessary for the process. Fresh normal serum by itself had very slight or no bacteriolytic power. Fresh immune serum had powerful and rapid effects. Heated immune serum had lost its power completely, but this was restored to it by the addition of the fresh normal serum. He noted, furthermore, that the specific nature of the bacteriolysis by the immune serum was unchanged after it had been inactivated by heat and reactivated subsequently by the normal serum. The inference was plain. Immunization of an animal incites the production, in the blood of this animal, of a "preventive" substance, which is moderately resistant to heat, and which is specific for the bacteria employed in the immunization. This substance cannot act upon the bacteria alone, however, but depends for its effective functionation upon the cooperation of another substance present universally in normal serum, the "bactericidal" substance, which is non-specific, corresponds to Buchner's alexin, and is apparently not increased by the process of immunization. These are the fundamental facts revealed by the early studies of Bordet, and they are stated in the present connection merely as experimental facts, without further elaboration of

[14] Metchnikoff. *Ann. de l'Inst. Past.*, Vol. 9, 1895.
[15] Bordet. *Ann. de l'Inst. Past.*, Vol. 13, 1899.

the later theoretical interpretation placed upon them by Bordet himself and by Ehrlich and his followers.

In the course of these studies Bordet [16] had used the immune serum produced in a goat by injection of cholera spirilla. As normal serum he had used guinea-pig serum, and the latter frequently contained a few blood corpuscles. He noticed that these corpuscles were frequently clumped in the goat serum and correlated this with the similar clumping (agglutination) of cholera organisms which he had noticed in this and other sera. In his incidental observation of the phenomenon of agglutination he had concluded that the living nature of the bacteria had no importance as far as their agglutination was concerned, dead organisms being as readily agglutinated as living.

Reasoning from this similarity between blood cells and bacteria in their behavior in serum, it occurred to him that the phenomena both of agglutination and of lysis might be expressions of general biological laws, not limited to bacteria. Accordingly he injected rabbit blood into guinea pigs, and examined the serum of animals so treated for its action upon rabbit corpuscles, in vitro. He found that the sera of "blood-immune" animals had acquired not only increased agglutinative power against the corpuscles injected, but had also acquired specific "hemolytic" powers, that is, the property of causing a solution of hemoglobin out of the red cells. (For the process of serum hemolysis does not consist of a complete dissolution of the red corpuscles, but rather in the liberation of the hemoglobin from the cell stromata.) The latter (shadow forms) can be recovered undisintegrated by the centrifugation of hemolyzed blood. The process, like that of bacteriolysis, was specific in that the hemolytic power was lost if the serum was heated to from 50° to 60° C., but could be restored undiminished by the addition of a little fresh normal serum, in itself possessing no hemolytic properties for the given species of cell. The specificity of the phenomenon again was seen to reside entirely in the heat-stable factor, the heat-sensitive or "alexin" factor being non-specific, and not increased during the process of immunization.

Observations related to those of Bordet concerning hemolysis were made independently, in the same year, by Belfanti and Carbone, who had observed that the serum of animals treated with blood cells of another species became toxic for this species, and extensive confirmation of the phenomenon of hemolysis was obtained, in the year following, by the work of von Dungern, and by that of Landsteiner.

After Bordet had thus established the important fact that hemol-

[16] See Bordet's own account in a "Résumé of Immunity"; "Studies in Immunity," Bordet, collected and translated by Gay, Wiley & Son, N. Y., 1909.

ysis was in every way analogous to bacteriolysis in that, like bacteriolytic sera, hemolytic sera could be inactivated by heat, but reactivated by the addition of small quantities of fresh normal serum, Ehrlich and Morgenroth [17] undertook an elaborate study of the mechanism of hemolytic phenomena, hoping thereby to elucidate the mechanism of lysis in general. For it is obvious that hemolysis lends itself far more easily to experimentation than does bacteriolysis, and, as we shall see, experiments on hemolysis can be made with a considerable degree of accuracy. Ehrlich and Morgenroth approached the investigation of the hemolysins from the point of view of the side-chain theory, formulated by Ehrlich in connection with his work on the toxins. According to this theory, it will be remembered, the hemolytic substances in the sera of animals treated with blood corpuscles represent the receptors or side chains of tissue cells. These receptors were originally integral chemical elements of the body cells, by means of which the cell became united to the injected erythrocyte (or bacterial) protein. Since union with the foreign substance blocked these receptors or side chains, thereby rendering them useless, they had been regenerated and, under the influence of immunization, regenerated in excess, cast off by the cell, and were now free in the blood stream as hemolysins (or bacteriolysins).

If this conception of the process was the correct one, Ehrlich and Morgenroth argued, the hemolytic substances of any immune hemolytic serum should possess specific chemical affinity, "haptophore groups," as they expressed it, for the blood cells which had been used in the immunization.

In order to show this, they inactivated at 56° C., by the method of Bordet, a goat serum which was hemolytic for beef blood, left it in contact with beef blood corpuscles for 15 minutes at 40° C., and then separated the cells from the supernatant fluid by centrifugation. To the blood cells they then added a little normal goat serum (by itself not hemolytic for beef blood) and found that complete hemolysis occurred. The subsequent addition of normal goat serum and beef blood cells to the supernatant fluid, however, resulted in no change.

In the following diagram we have tried to represent this basic experiment, giving the facts only of the experiment without using any of the usual symbols which imply agreement with a theory.

EXPERIMENT TO SHOW THAT THE ANTIGEN (IN THIS CASE RED BLOOD CELLS ABSORBS THE SPECIFIC HEAT STABLE ANTIBODY OUT OF THE IMMUNE SERUM.

In a test tube $\left\{\begin{array}{l}\text{4 c. c. of 5 per cent. emulsion of washed beef blood.}\\ \text{1 c. c. of inactivated blood serum of a goat treated with beef}\\ \quad\text{blood.}\end{array}\right.$

[17] Ehrlich and Morgenroth. *Berl. klin. Woch.*, Nos. 1, 21, and 22, 1900.

These substances are left together at 37.5° C. for one hour and then centrifugalized into:

I

Sediment of Corpuscles.—To this are added 4 c. c. salt solution and 0.8 c. c. fresh normal goat serum, by itself not hemolytic for beef corpuscles.
Result = *Complete hemolysis.*

II

Supernatant Fluid Containing the Serum and Salt Solution.—To this are added washed beef corpuscles and 0.8 c. c. fresh normal goat serum.
Result = *No hemolysis.*

Summarized, together with the facts we have already outlined, this basic experiment has the following significance: the fresh serum of the goat, previously injected ("immunized") with the beef blood, possessed the property of dissolving the hemoglobin out of beef corpuscles, viz., hemolyzing them. Heating this serum to 56° C. for 20 minutes, as Bordet has shown, deprives the serum of all hemolytic power, i. e., inactivates it. The addition of a little fresh goat serum, in itself inactive, completely reactivates the hemolytic properties of the heated immune serum. So far, as we have already seen, this shows that hemolysis is a dual process in which a heat-sensitive and a heat-stable substance coöperate, neither of them capable of producing lysis by itself. The heat-sensitive ingredient, corresponding to Buchner's "alexin," is present in normal serum, and, as Bordet [18] and von Dungern [19] had shown, is not increased in the process of immunization, and is apparently not specific. The heat-stable substance, therefore specific and increased in immunization, must represent the receptors, overproduced and cast off into the circulation. And, as Ehrlich and Morgenroth have now shown in the experiment just described, this heat-stable element is actually bound to the red corpuscles, and renders them susceptible to the action of the heat-sensitive substance in the normal goat serum. And furthermore, in attaching to this heat-stable element, the blood cells have removed it from the solution. For we have seen, in the experiment, that addition of corpuscles and normal serum to the supernatant fluid resulted in no hemolysis, showing that the third necessary element, originally in the mixture, had been carried down with the red cells.

In these and other experiments then, it was shown that only the heat-stable substances could be fixed by the red cells, and this even at temperatures at or about 0° C. (a fact which indicates the strong affinity between the two substances), while the heat-sensitive "alexin," which Ehrlich now called *"complement,"* could not attach directly to the red cells. For if such complement, in the form of fresh serum, was added to washed red blood cells, and the mixture after standing at 40° C. for some time was centrifugalized, the com-

[18] Bordet. *Ann. de l'Inst. Past.*, Vol. 12, 1898.
[19] v. Dungern. *Münch. med. Woch.*, No. 20, 1900, p. 677.

plement remained in the supernatant fluid, as could be easily shown by an experiment such as the one represented in the following protocol.

EXPERIMENT TO SHOW THAT COMPLEMENT OR ALEXIN IS NOT ABSORBED BY UNSENSITIZED CELLS

Mixed in a test tube { 4 c. c. of 5 per cent. emulsion of washed beef blood.
0.8 c. c. of fresh normal goat serum (alexin or complement), not, by itself, hemolytic for beef blood.

These substances are left together at 37.5° C. for one hour, then centrifugalized into:

I	II
Sediment of Cells.—To this is added *inactivated serum* of immune goat which would cause hemolysis if alexin were present.	*Supernatant Fluid* (salt solution and serum).—To this is added washed beef blood and inactivated serum of immune goat containing heat stable element.
Result = *No hemolysis.*	Result = *Complete hemolysis.*

Although, therefore, the red cells bind the thermostable specific antibody of the immune serum and not the complement or alexin, it was shown both by Bordet and by Ehrlich and his collaborators that the red cells, after absorption of the thermostable substance, when exposed to the action of the complement, were not only disintegrated by hemolysis but, in the process, fixed or attached the complement, so that this was no longer available for further activation of other sensitized cells.

The fact that the alexin or complement is used up during processes of lysis, as first described by Bordet, Ehrlich, and others, has recently been made the subject of repeated investigation, since this is out of keeping with the general enzyme or fermentlike nature of complement indicated by many of its other properties.

Muir,[20] who studied the conditions thoroughly, comes to the conclusion that the complement is in truth used up in hemolysis, but that it does not always disappear completely, this depending upon the relative amount of sensitizer or amboceptor present. (He confirms the quantitative ratios between the two substances found by Morgenroth and Sachs in hemolytic reactions, a subject discussed by us in another place.)

Liefmann and Cohn,[21] in a more recent publication, have come to different conclusions. They believe that the disappearance of free complement from hemolytic complexes is not due to its chemical

[20] Muir. *Lancet,* Vol. 2, 1903, p. 446.
[21] Liefmann and Cohn. *Zeitschr. f. Immunitätsforsch. Or.,* Vol. 8, p. 58, 1911.

union with the sensitized cells in the process of hemolysis, but is due rather

(1) to a fixation by the products of hemolysis (stromata, etc.) after the reaction is accomplished,

(2) to dilution, and

(3) to weakening because of prolonged preservation in dilute solution at 37° C.[22]

Theoretically this is of considerable importance if confirmed, since it would bear out strongly the conception of complement as a true enzyme or ferment. From the point of view of the practical utilization of complement fixation for various purposes it makes little difference, since here the disappearance of complement is the essential thing, irrespective of whether this occurs in the course of its activity or because of fixation by the products of its own action.

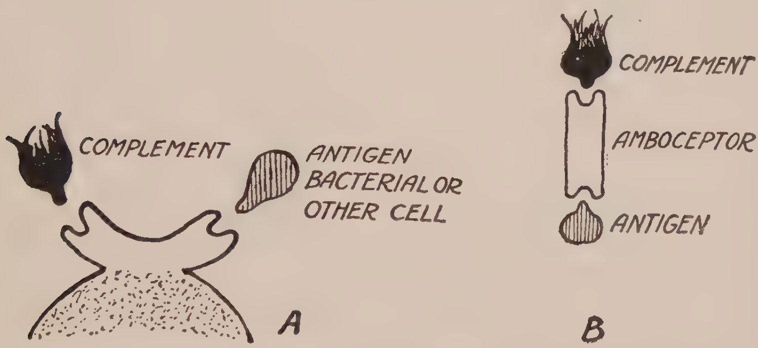
We now have the basic principle of hemolysis; facts which can easily be shown to hold good for bacteriolysis and for the bactericidal processes even when no actual solution takes place. Briefly reviewed, these facts are as follows: The antigen (blood cells, bacterial cells, etc.) undergoes hemolysis or bacteriolysis when acted upon by two factors, one a thermostable substance, specific and increased during immunization, the other a thermosensitive substance present in fresh serum, not increased [23] by immunization of the animal with the antigen and not specific. The specific thermostable substance becomes united with or fixed to the antigen regardless of the presence or absence of the thermosensitive alexin or complement, and with such avidity that the union takes place even at 0° C. The alexin or complement, however, cannot enter into relation with the antigen unless this has been rendered susceptible to it by attachment to the thermostable specific substance. When this has taken place, union with complement occurs, but only at temperatures above 0° C. (the speed and completeness of the union increasing as the temperature approaches 40° C.), and the result of the union is lysis or, in the case of bacteria not easily soluble, the bactericidal effect.

The "Amboceptor" Conception.—It appears from the preceding that the thermostable hemolytic antibody must, of necessity, unite with the red cell before the complement or alexin can exert its action upon it. Ehrlich conceives this process as a mediation on the part of the heat-stable substance between the antigen and the alexin or complement. The heat-stable body, which he calls "amboceptor," because of its assumed mode of action, possesses two combining groups—one the "cytophile," by means of which it is anchored to the sensitive cell, the other the "complementophile," by means of which it exerts affinity for the complement. The original cell re-

[22] In the ordinary dilution used in Wassermann tests, the unit of complement employed may deteriorate entirely within several hours at 40° C.

[23] Bordet. *Ann. de l'Inst. Past.*, Vol. 12, 1898. Confirmed by v. Dungern, *Münch. med. Woch.*, No. 20, 1900.

ceptor, from which such an "amboceptor" takes its origin, is one which not only can combine with the antigenic substance offered for assimilation, but which also possesses another atom group by means of which it can enlist the aid of the digestive ferment of the blood, the alexin or complement. Cast off into the blood stream, as a result of overregeneration, it now appears as a "double" receptor, which can form a link between the antigen and the complement or alexin.



Schematic Representations of a Receptor of the Third Order.
Ehrlich's conception of the relationship of antigen, amboceptor, and complement in the bactericidal and hemolytic process. In A the receptor is still a part of the body cell, in B it has been overproduced, and is free in the circulating blood.

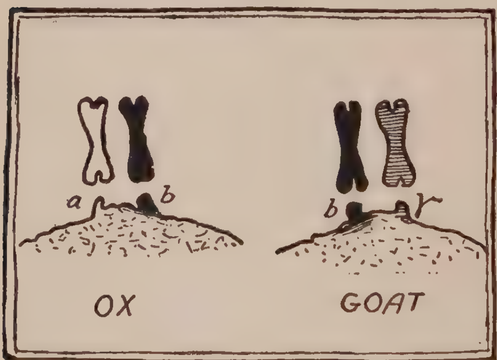
In his general scheme of diagrammatic representation of these processes Ehrlich refers to the "amboceptors" as "haptines" of the third order.

Now it is quite plain, from the extreme specificity which results when an animal is immunized with any given variety of blood cells or bacteria, that there must be as great a variety of such amboceptors as there are different antigens, and indeed an animal immunized with two or more antigens may simultaneously contain in its blood serum a corresponding number of separately recognizable amboceptors.

This assumption of the multiplicity of "amboceptors" in the same serum is, of course, forced upon us by the fact of specificity, and the frequently repeated observation that the same serum may contain heat-stable lytic antibodies against a variety of antigens, each antigen absorbing out of such a serum that antibody only which specifically reacts with it. This fact has, of course, never been denied, and it is a frequent misunderstanding of the views of Bordet, which will be discussed directly, to assume that he has combated the "multiplicity of amboceptor" in the sense just outlined. Ehrlich and Morgenroth, however, have expressed themselves in favor of the

conception of a multiplicity of "amboceptor" not only in this sense, but as occurring in response to immunization with one and the same antigen.

Ehrlich and Morgenroth [24] assume that any cellular antigen, blood or bacterial cell, substances of great complexity of chemical structure, must necessarily be possessed of a large number of different side chains or receptors. When immunization is practiced with such cells a correspondingly varying number of different amboceptors must result. They found, for instance, that when rabbits are immunized with ox blood, the resulting antiserum is capable of producing hemolysis not only of ox blood but of goat's blood as well, though to a lesser degree. They conclude from this that the hemolytic action of the serum must be referred to the presence of at least two kinds of amboceptor, especially since repeated experiments with different anti-ox-blood sera showed that there was no regularity in the proportions of hemolysins for ox and goat blood, respectively. This opinion they further fortify by showing that exposure of the serum to ox blood deprives it of all its hemolysins, both those for ox and those for goat's blood, whereas absorption with goat's blood alone removes the specific goat's blood hemolysins only. They translate their understanding of the conditions to graphic form by the following diagram: [25]



Schematic Representation of Ehrlich and Morgenroth's Conception of the Complex Structure of an Antigen.

(After Ehrlich and Morgenroth. *Berl. klin. Woch.*, Vol. 38, 1901.)

If ox blood is injected, α and β receptors being present, α and β amboceptors are formed, and ox blood can consequently anchor both amboceptors. The presence of β receptors in goat's blood also explains the modern hemolysis of this blood by the antiserum, but lacking the α receptors which, in this case, represent the larger proportion, these blood cells cannot remove all the amboceptor for ox blood out of the serum. The example given, of course, represents the simplest assumed case, and Ehrlich and Morgenroth believe that the same blood or bacterial cells may possess an entire series of such receptors, some of them being dominant for the given

[24] Ehrlich and Morgenroth. *Berl. klin. Woch.*, Nos. 21 and 22, 1901.
[25] Ehrlich. "Gesammelte Arbeiten," p. 147.

species, others being merely secondary or "partial," in varying proportions.

If we grant the fundamental premises of Ehrlich respecting the "double receptor" or "amboceptor" nature of the specific antibody and its mediation between antigen and complement by means of a cytophile and a complementophile receptor, certain logical consequences of this conception suggest themselves, which, in their many ramifications, have been the subject of much investigation. And although many phases of these researches are no longer commonly accepted, some, indeed, being untenable in the light of more recent discoveries, the influence of this work upon the development of immunology has been so important that it must be briefly reviewed in order that controversial questions may be given the critical discussion which they merit.

The comparison of the action of hemolytic sera with that of ferments, and the possibility of producing antiferments by the injection of the ferments into animals, obviously suggests a similar induction of antihemolysins by the treatment of animals with lysins. This, we have seen, was the method employed by Ehrlich and Morgenroth in their studies of the causes of the failure of autolysin formation in goats. They extended this work with the purpose of ascertaining whether or not there were differences in the structure of the cytophile groups of the various amboceptors formed when various animals were injected with any given species of red blood cells. After obtaining a strong hemolytic serum by injecting ox blood into a rabbit, they treated a goat with the inactivated serum of this rabbit. The result was that the serum of the goat so treated, when mixed with ox blood cells and the hemolytic serum, prevented the sensitization of the cells by the hemolysin. They then measured the neutralizing power of such an "anti-amboceptor" or "anti-sensitizer" against a variety of hemolytic sera produced with ox blood in different animals and found that, while this "anti-amboceptor" neutralized the hemolytic action of an antiserum produced in rabbits, it had but an indifferent or entirely ineffective neutralizing power upon similar ox blood hemolysins derived from goats, geese, dogs, rats, or guinea pigs. They concluded from this that, although these various lysins had been produced in the different animals by the injection of the same antigen, viz., ox blood, and possessed affinity for the ox blood in consequence, they must necessarily differ from each other in some way, since they were not equally neutralized by the same antilysin. It seemed to them that the difference in such cases must depend upon variations in the structure of the cytophile group of the amboceptor, a conclusion which they based upon the foregoing experiments and sought to support by the following reasoning: When an animal is treated with sensitizers or amboceptors, they reasoned, these bodies react with the tissue cells by means of the

cell-receptors. These receptors are then overproduced and extended into the circulation as free atom-groups.

They now act as "anti-amboceptor," free in the serum, but are in structure merely overproduced cell receptors, identical with those which originally united *on* the cell with the injected amboceptor.

Ehrlich and Morgenroth,[26] therefore, believed that the neutralization of the amboceptor by the antilysin depended upon a union of the latter with the "cytophile" group of the former, preventing its subsequent union with the red cells. And since one and the same antilysin did not thus invalidate the action of all the amboceptors for ox blood (derived from different animals), they concluded that these "amboceptors" must possess different "cytophile groups."

SCHEMATIC REPRESENTATION OF EHRLICH AND MORGENROTH'S CONCEPTION OF THE NEUTRALIZATION OF A HEMOLYTIC SERUM BY ANTILYSIN OR ANTIAMBOCEPTOR, REACTING WITH THE CYTOPHILE GROUP. (Ehrlich and Morgenroth, *loc. cit.*)

This conception, as we shall see, has become untenable.

That this conclusion of Ehrlich and Morgenroth is not correct seems to follow the subsequent work of Bordet.[27] He demonstrated that it is not necessary to inject animals with specific hemolytic sera in order to obtain antilytic sera, but that the same object may be attained by injecting animals with the normal serum of an untreated animal. Moreover, if an "antisensitizing" serum so produced was added to corpuscles which had already absorbed "amboceptor," it prevented the subsequent union of these sensitized cells with alexin or complement. From this it becomes clear that, in the first place, the antisensitizer or anti-amboceptor cannot be identical with the cell receptors of the corpuscles, and, further, that the inhibition of the hemolysis which such an antisensitizer exerts, cannot be due to union with the "cytophile" group. This both contradicts the Ehrlich conception of the mechanism of "anti-amboceptors" and invalidates his argument, in this instance, in favor of the plurality of the amboceptors produced by the injection.

Bordet's experiments were later confirmed by Ehrlich and Sachs,[28] who admit the error of the former "anticytophile" interpretation of Ehrlich and Morgenroth's experiments, but they still maintain that Bordet's experiments do not disprove the conception of an "amboceptor" or "Zwischenkörper" of Ehrlich. They claim that

[26] Ehrlich and Morgenroth. *Berl. klin. Woch.*, No. 22, 1901, p. 600.
[27] Bordet. *Ann. de l'Inst. Past.*, Vol. 18, 1904, p. 593.
[28] Ehrlich and Sachs. *Berl. klin. Woch.*, No. 19, 1905.

Bordet's results merely prove that the anti-amboceptor or antisensitizer is "anticomplementophile" instead of "anticytophile."

The principles involved we will discuss in another place in connection with Moreschi's analysis of the "anticomplements." However this may be, we may conclude that Ehrlich and Morgenroth's differentiation of amboceptors or sensitizers by the cytophile group is no longer valid.

The studies of Bordet on the antisensitizers (anti-amboceptor) had important results apart from their refutation of Ehrlich and Morgenroth's opinion. In addition to showing that such antisensitizer did not represent cell receptors identical with those that anchored the sensitizer (amboceptor) to the red blood cells, his experiments revealed the fact that such an antisensitizer neutralizes unspecifically various specific sensitizers as well as normal antibodies in the serum of the same animal; and this showed that there is no necessity of assuming a variety of specific antisensitizers, as had been done by the German workers.

As regards the multiplicity of amboceptor or sensitizer, however, though the proof of this, by means of anti-amboceptors, has had to be abandoned, as we have seen, there is still a great deal of evidence advanced in favor of such an assumption. The chief support for such an opinion is found in the "group reactions" among bacteria, similar to those observed for blood cells by Ehrlich and Morgenroth, and described above (see page 166). For it is frequently observed that the antibodies produced by immunization with one species of bacteria may have a certain though lesser degree of action upon other related forms, these in turn absorbing only a part of the amboceptor out of the serum, while the species originally used for immunization takes out all the amboceptor present. Considering the great chemical complexity of the bacterial or tissue cells, moreover, we may well expect such multiplicity. And it is, indeed, entirely reasonable to suppose that a structure as complex as the bacterial cell may contain a number of antigens and consequently give rise to a number of sensitizers which differ in that each is specific for its particular antigen only. This is merely a restatement of the phenomenon of specificity and has, as a matter of fact, no modifying influence on the general principles involved.

Multiplicity or Singleness of Alexin.—From the point of view of a general understanding of the processes of immunity, however, the question of multiplicity of sensitizer is not so fundamentally important as is the similar controversy which has been waged regarding the unity or multiplicity of alexin or complement. Here again there has been some misconception as to the meaning of those who maintain the unity of alexin. Neither Bordet, nor anyone else familiar with experimental conditions, has ever maintained that the alexins of different animals were functionally identical.

It is a well-known fact that the fresh blood sera of various animal species differ from each other considerably in their power to activate bactericidal or hemolytic systems. In regard to hemolysis, fresh guinea-pig serum is very powerful in activating many sensitized blood-cell complexes, but weak in activating sensitized guinea-pig corpuscles. Often one finds that the alexin of an animal is entirely impotent or but weakly capable of producing hemolysis of the sensitized cells of its own species, though this is not a general rule.

Again, even without such species relationship, a given alexin may be very weak for certain complexes and strong for others. The alexin of horse blood can even be fixed to sensitized cells [29] without producing much, if any, hemolysis.[30] An alexin which may be strong for a given hemolytic complex may be weak for certain bactericidal complexes, or *vice versa.* Thus there is a large mass of evidence which shows that no two alexins are exactly alike, though the difference between them can, of course, be defined functionally only.

The difference between the opinions of Ehrlich and his school on the one hand, and the followers of Bordet, on the other, revolves not about this point, upon which all agree, but about the question of whether one and the same serum may contain more than one alexin or complement. Ehrlich and Morgenroth [31] and Ehrlich and Sachs [32] have brought forward evidence from which they deduce the existence of a number of different alexins or complements for hemolytic complexes in the same serum. The earlier experiments of Ehrlich and Morgenroth on this question were carried out by means of the filtration of normal goat serum through Pukall filters; [33] in these it appeared that the serum which passed through the filters was complementary for sensitized guinea-pig cells, while that part which had, in the original serum, hemolyzed sensitized rabbit cells was left behind. Similar differentiation of complement they later based upon experiments with anticomplementary sera which, they showed, did not equally neutralize all the complementary functions of a serum.

In support of their contention Neisser [34] described two complementary substances in rabbit serum, the one active for bactericidal complexes, the other for hemolytic, and similar experimental evidence has been brought forward by Wassermann [35] for guinea-pig and by Wechsberg [36] for goat serum.

[29] For the sake of clearness it may be repeated here that by sensitized cells we mean cells which have absorbed specific "amboceptor" or "sensitizer," and have thereby become amenable to the action of alexin or complement.

[30] Browning. *Wien. klin. Woch.,* No. 15, 1906.

[31] Ehrlich and Morgenroth. *Berl. klin. Woch.,* No. 31, 1900.

[32] Ehrlich and Sachs. *Berl. klin. Woch.,* No. 21, 1902.

[33] Sachs. *Berl. klin. Woch.,* Nos. 9 and 10, 1902.

[34] Neisser. *Deutsche med. Woch.,* 1900, p. 790.

[35] Wassermann. *Zeitschr. f. Hyg.,* 37, 1901.

[36] Wechsberg. *Zeitschr. f. Hyg.,* Vol. 39, 1902.

The evidence advanced by these writers is based chiefly on experiments in which it was found that a normal serum which possessed both bactericidal and hemolytic powers could be deprived of the complement for one or the other of these activities only, by absorption with the respective cells. In addition to this, Ehrlich and Morgenroth, Ehrlich and Sachs,[37] Wendelstadt,[38] and others, claimed to have differentiated various complements in the same serum by careful heating, by the action of weak acids or alkalis, or such methods as the digestion of sera by papain.

As a rule, these experiments have been carried out with normally hemolytic serum and unsensitized cells, though in certain cases Ehrlich has employed sensitized cells; but whenever this was done exposure to complement for purposes of absorption has been for much briefer periods than when normal serum was used. This point is significant when we come to consider the objections to the interpretation of the preceding experiment in favor of a plurality of complement, objections raised chiefly by Wilde [39] and by Bordet.

Wilde refuted particularly the experiments of Neisser, who claimed that the absorption of fresh rabbit serum with anthrax bacilli deprived this serum only of its bactericidal but not of its hemolytic complement. Wilde showed that, if a sufficient excess of anthrax bacilli (or in given cases of typhoid bacilli or cholera spirilla) were added, both bactericidal and hemolytic complement could be absorbed from normal serum. He concludes that there is actually only one alexin present, but that the red cells and anthrax bacilli differ in their susceptibility to this alexin (or, in other words, that the sensitization of these cells by the normal serum is unequal, a conclusion which seems rational in view of the fact, now well known, that one and the same complement may differ greatly in the degree of its activity upon different sensitized complexes.

Bordet has analyzed the conditions in a similar way. He found that absorption of normal serum with unsensitized cells rarely deprived this serum of all of its alexin, even when these cells were used in considerable amounts. This he attributed to the feeble sensitization of the cells. If, however, strongly sensitized cells were added to such a normal serum, all the alexin would be taken up. He refers the phenomenon of specific alexin absorption, observed by previous workers, to insufficiency in the perfection of sensitization on the part of the cells used in the preliminary exposure; and subsequent work with complement fixation seems to bear him out.

Most of these arguments, though they seem to us perfectly valid in the light of the experimental facts, have been answered by Ehrlich and his school by the assumption of the existence of so-called "poly-

[37] Ehrlich and Sachs. *Berl. klin. Woch.*, Nos. 14 and 15, 1902.

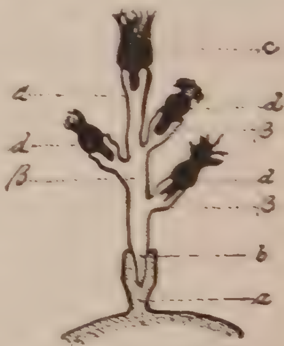
[38] Wendelstadt. *Centralbl. f. Bakt.*, I, Vol. 31, 1902.

[39] Wilde. *Habilitations Schrift*, Munich, 1901. Also *Berl. klin. Woch.*, No. 34, 1901.

ceptors." Ehrlich now admits that the amboceptors cannot be shown to differ from each other. However, he does not believe that differences in the intensity of sensitization explain variation in the functional efficiency of different complements upon sensitized cell complexes, nor does he accept, for proof of this, the fact that complement may be entirely absorbed out of a serum by a complex, even though the complement may be comparatively inefficient as an activator in the given case. He assumes that the sensitizer or "amboceptor" may possess a number of complementophile groups (polyceptors), by means of which a number of different complements may become active in the given case. Thus, although such a polyceptor, of course, is capable of uniting with the complement which activates the dominant complement, it is capable also of union with a number of other complements which have slight or no functional action whatever—the non-dominant complements. This opinion is rendered diagrammatic by Ehrlich and Marshall [40] in the following way:

EHRLICH AND MORGENROTH'S CONCEPTION OF THE ACTION OF ANTICOMPLEMENT.

A. Scheme of Hemolysis.
B. Action of Anticomplement upon Hemolysin.
b. = blood cell, c. = complement, i. = immune body, a. = anticomplement.
The complementoids are not included in the scheme, since in this case they are without influence.



POLYCEPTOR ACCORDING TO EHRLICH AND MARSHALL

(a) Receptor of the Cell.
(b) Haptophore Group of the Amboceptor.
(c) Dominant Complement.
(d) Secondary Complements.
Complementophile groups of the Amboceptor:
(1) for the Dominant Complement.
(2) for the Secondary Complement.
(After Ehrlich and Marshall, *Berl. klin. Woch.*, No. 25, 1902.)

If one carefully considers the reasons advanced for the assumption of the existence of such polyceptors it does not seem that they are sufficiently forcible to lead one to desert the much simpler explanation of Bordet.

Related to the problems discussed in connection with the production of "antiamboceptors" or "antisensitizers" are those which have arisen regarding the existence of "anticomplement" or "antialexins."

[40] Ehrlich and Marshall. *Berl. klin. Woch.*, No. 25, 1902.

Ehrlich and Morgenroth claimed that, by the injection of active horse serum into a goat, they had obtained substances in the goat serum which neutralized horse complement. They believed that the "anticomplements" thus produced neutralized the complement by uniting with its haptophore group, thus preventing its combination with the "complementophile group" of the amboceptor. This was their conclusion because they found that the "anticomplementary" serum exerted no protective influence upon sensitized cells, when these were exposed to the serum and then removed, but that it protected against hemolysis when added to the cells together with the complement. There was apparently no union of the protective substance with the "complementophile" group of the amboceptor, but the protecting substance *did* act in direct antagonism to the complement itself.

From the fact that similar anticomplements could be produced when inactivated serum was injected into animals, they concluded that, on inactivation, there was not a complete destruction of the complement, but that during the process of heating the zymophore group of the complement only was injured, the "haptophore group," by means of which union to the tissue elements would take place, and through which, therefore, specific antibody production would be incited, remained intact. Such altered complement they speak of as "complementoid."

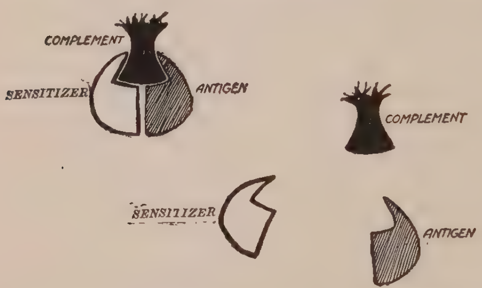
Bordet has made similar observations upon the production of anti-alexins by the injection into animals both of active and of inactive serum, but in the light of further researches, which will be discussed in connection with the problems of alexin-fixation, chiefly those of Moreschi and of Gay, we are forced to the conclusion that the existence of true anticomplements is by no means certain, and that the older evidence in their favor is found to be unconvincing at the present time.

In the preceding paragraphs we have emphasized the conceptions of the cytolytic phenomena formulated by Ehrlich and his followers, and although we have brought out, whenever possible, the objections of other investigators to many of these opinions, we have not yet followed out in a systematic manner the reasoning of any of Ehrlich's opponents. In opposition to the views of his school the leading position has been taken by Bordet, who, after all, furnished in his investigations the fundamental facts which have led to a comprehension of the cytolytic processes. In explaining Bordet's views we can do no better than to follow out his own exposition as set forth in his article, "A General Résumé of Immunity," [41] published with a collection of his papers. He expresses himself, in substance, as follows:

That the antigen, in the form of bacteria, blood cells, or cells of

[41] "Studies in Immunity," by Bordet and collaborators. Gay, Wiley & Sons, N. Y., 1909.

any other nature, meets in the body of the treated animal a "receptor" complex with which it unites is, of course, plain and agreed to by everyone. That the antibody produced by the tissues in response to such union of antigen with receptor is a direct product of the cells containing the receptors is likely. It is by no means certain, however, or, at any rate, it has never been experimentally demonstrated, that, as Ehrlich maintains, the antibody is identical with the original receptor by which the antigen was fixed or anchored to the tissue cell. It might be assumed with equal justice that the cells of the immunized animal could build up a new substance, not identical with the receptors, in consequence of stimulation by the antigen. It is also by no means certain whether the injected antigen reacts with the body cells themselves or with the normal antibodies which we know to exist in many cases. Thus the blood serum of goats may normally often contain hemolysins against rabbit corpuscles. Is it not reasonable to suppose that possibly these may furnish the point of attachment and the source of further antibody production when rabbit cells are injected into goats? In criticism of Ehrlich's assumption of the mode of action of heat-stable lytic antibody, Bordet very justly maintains that *no proof whatever exists of the "amboceptor" nature of this substance.* All that is certain is that the stable substance must unite with the antigen before the alexin or complement can exert its action upon it or be fixed by it. There is no entirely valid proof of the existence in this antibody of a "complementophile" and a "cytophile" group, and no satisfactory instance has been observed in which alexin has united with a heat-stable antibody which has not previously been united with an antigen.[42] All that has been shown is that the antigen, together with its specific antibody, forms a complex which has an avidity for alexin, a complex which is "endowed with properties of absorption for complement which neither of its constituents alone possesses." Bordet speaks of the "amboceptors," therefore, as "sensitizers,"



Schematic Representation of Bordet's View Concerning the Inability of Complement to Unite with Either Antigen or Sensitizer Alone and Its Ability to be Fixed by the Complex Formed When the Antigen is Sensitized.

Compare this figure with that representing Ehrlich's conception of the same process.

[42] Refer also to the discussion of the conglutinins at the end of this chapter.

meaning by this that the antigen, by union with its antibody, is sensitized to the action of the alexin. The term "sensitizers" in no way, therefore, implies a preconceived notion, experimentally unproved, of the mode of action or structure of the sensitizer. Since we have graphically explained Ehrlich's opinions, a similar diagrammatic representation may be permitted of Bordet's opinion of the same process of union of antigen and heat-stable antibody with the consequent development of alexin-fixing property.

In this diagram the ability to absorb or unite with complement becomes evident only after a complex has been formed by the union of the two elements, antigen and antibody. The diagram must not be assumed to mean that the notch into which the complement fits symbolized necessarily an "atom group," but merely expresses the idea of "ability to absorb alexin," not assuming that this ability is either chemical affinity by means of a definite atom group or a mere physical change of molecular equilibrium permitting a specific complement absorption.[43]

"Zone" Phenomena.—It will be seen from the preceding that the controversy between Ehrlich's "amboceptor" conception and the "sensitization" idea of Bordet turns largely upon the existence of a so-called complementophile group of the thermostable antibody. For if it were the case that this antibody possessed an atom group which permitted it to unite with alexin, independent of previous union with antigen, it would go far to support Ehrlich's view. One of the strongest arguments brought into the field in favor of such an occurrence by Ehrlich's followers is the phenomenon of Neisser and Wechsberg, which is usually spoken of as "complement deviation" (Komplement Ablenkung).

In order to make the conditions underlying this phenomenon clear, it will be of advantage to consider for a moment the methods of determining quantitatively the amount of bactericidal antibody (sensitizer amboceptor) in any given immune serum, since it was in working with such titrations that Neisser and Wechsberg made their observations.

In carrying out such measurements, it is customary to add in series, to constant amounts of bacteria, varying amounts of inactivated antiserum and constant amounts of complement or alexin.

[43] The diagram on page 174, though possibly not expressing with absolute accuracy the idea of sensitization, was devised because it will remove what seem to the writer frequent misconceptions of Bordet's views. Statements are found in the literature which imply (Ehrlich, "Kraus und Levaditi Handbuch," Vol. 1, p. 8) that Bordet assumes "dass das Komplement direkt an die Zelle angreift," and deny that there is experimental evidence to support this. It is perfectly true that there is no evidence to show such "direktes Angreifen" upon the unaltered cell, but there is evidence that this union takes place after the cell has absorbed the antibody, and no satisfactory evidence to show that the thermostable body is an intermediary, that is, forms a link as conceived in the amboceptor idea.

These mixtures are set away in the thermostat for 3 to 4 hours, are then mixed with agar and plates are poured. The colonies which develop will give an indication of the number of bacteria killed in each mixture when compared with similar plates poured from tubes in which the same original amounts of bacteria had been mixed with alexin alone. The following table will exemplify such a test:

Typhoid bacilli	Typhoid antiserum inactive	Alexin	Result in colonies after 3 hours' incubation
Constant quantity	.1 c. c.	.07 c. c.	Many thousand
Constant quantity	.01 c. c.	.07 c. c.	Many thousand
Constant quantity	.005 c. c.	.07 c. c.	150 colonies
Constant quantity	.001 c. c.	.07 c. c.	200 colonies
Constant quantity	.0005 c. c.	.07 c. c.	800 colonies
Control I, constant quantity	....	.07 c. c.	Many thousand
Control II, constant quantity	....	....	Many thousand

In this table it is noticeable that, although there has been considerable bactericidal action in the mixtures in which 0.005, 0.001, and 0.0005 c. c. of antiserum were used, the mixtures in which as much as 0.1 and 0.01 c. c. were present, and in which one would naturally expect a still greater antibacterial action, the contrary occurred.

This surprising and curious phenomenon, showing that an excess of antibody could actually be harmful to the bactericidal complex, was explained by Neisser and Wechsberg by the following reasoning. In tests like the one given above a limited amount of bacteria and alexin has been mixed with the enormous amount of antibody represented in the immune serum. Although bacteria can absorb more of this antibody than is necessary for their solution or destruction, nevertheless the higher concentration given in the table will contain quantities of "amboceptor" so far in excess of the amount that can be absorbed that much of it must remain free in the fluid. Now this amboceptor, possessing a complementophile group, is able to anchor complement or alexin as well as that which has become united with the bacteria. In consequence, there being only a limited amount of complement, some of this is deviated from the amboceptor-antigen complexes by the free amboceptor, and is, in consequence, ineffective so far as bactericidal action is concerned. In the higher dilutions of the antiserum, in which no such excess is present, the complement will be concentrated upon the "attached" or "anchored" amboceptor, and greater efficiency will result.

As to the accuracy of the observations of Neisser and Wechsberg there can be no question, and everyone who has occasion to carry out bactericidal tests with any frequency is sure to meet with the phenomenon again and again. But their explanation, which involves

the assumption of union between free sensitizer or amboceptor, and alexin or complement, without the participation of antigen, cannot be accepted since, search as we may, through the extensive experimentation that this problem has inspired, there is no instance on record in which indisputable evidence of such an occurrence has been advanced. On the contrary, there is a mass of satisfactory evidence available which indicates clearly that amboceptor or sensitizer alone cannot absorb alexin, and the Neisser-Wechsberg explanation seems consequently to be merely an interesting and cleverly conceived but improbable possibility.

What, then, is the explanation of the diminution of bactericidal effect in the presence of an excess of sensitizer? We will see that, in the study of agglutinin and precipitin reactions, phenomena exactly analogous to the Neisser-Wechsberg effect have been noticed, in the case of the agglutinins, the so-called "pro-agglutinoid" zone being a case in point. For these phenomena, as well as for that of Neisser and Wechsberg, explanations have been advanced by the Ehrlich school, similar in principle in that they all depend upon more or less arbitrary assumptions regarding affinity between the reacting bodies. Such explanations, though not outside the realm of possibility, have, however, lost much force since it has been recognized that the reactions between serum antibodies and their antigens, in general, take place according to laws far more closely analogous to those governing reactions between colloids than to those governing chemical reactions in which the laws of definite proportions can be applied. And, indeed, the reacting substances in antigen-antibody complexes are, beyond doubt, of the nature of colloids. Now, in many precipitations resulting when two colloids are mixed, an excess of one or the other factor will completely inhibit the occurrence of the precipitation; the reaction taking place only when definite proportions between the reacting bodies are present. The occurrence of such inhibition zones, due to an excessive concentration of one reagent, can be shown for agglutination and precipitation, exactly as it can in ordinary colloidal reactions, and it is more than likely that the Neisser-Wechsberg phenomenon is merely an example of a similar phenomenon.

This inhibiting effect upon reactions produced by excess of either antigen or antibody has been particularly noticeable in complement or alexin fixation reactions which are considered in a separate section below. In this place, however, it is important to discuss the so-called zone phenomena as observed in researches done upon this occurrence in connection with alexin fixation experiments. Dean [44] has extensively investigated the matter in parallel experiments in which precipitation reactions and complement fixation reactions were compared. He found that an optimum precipitation or an

[44] Dean. *Zeit. f. Immunitäts.*, 13, 1912, 84.

optimum complement fixation could be obtained only when very definite relative proportions of antigen and antibody were used. If either of the reacting substances was increased from the amount in the optimum mixture, both reactions were diminished in intensity. Within certain limits, if both were diminished proportionately, intense reactions could still be obtained. Work of entirely similar significance has recently been done in our laboratory by J. T. Parker with residue antigens such as those described at the end of the complement fixation section. These investigations definitely contradict the explanation of Neisser and Wechsberg and bring such reactions into very close analogy with what we generally speak of as colloidal reactions. They will be taken up again in our discussion of the precipitation reaction in connection with which they have been most carefully studied.

Looked at from this point of view, far from supporting the supposition of a separate complementophile group and therefore of the "amboceptor" nature of the heat-stable lytic antibody, the Neisser-Wechsberg phenomenon indeed becomes rather a strong argument in favor of Bordet's views, and against those of Ehrlich. For, by introducing the analogy between the lytic and bactericidal processes with colloidal reactions, it takes away much force from the supposition that antigen-sensitizer alexin reactions take place according to laws of definite proportion, an idea which still underlies, though somewhat loosely, many of the more important views of antigen-antibody reactions as conceived on the basis of the analysis made by Ehrlich and his school.

Gay has suggested also that the Neisser-Wechsberg phenomenon may well be explicable on the basis of the fixation of complement by precipitates. In a succeeding section we will discuss the fixation of alexin, which occurs when a dissolved protein is brought together with its specific antiserum. It is not impossible that this may occur when bacterial emulsions, from which a small amount of bacterial protein may well go into solution, are brought together with antiserum in concentration. Under such conditions a reaction might readily occur which would lead to the fixation of alexin and its consequent deviation from the sensitized bacteria.[45]

Quantitative Relationship of Sensitizer and Alexin.—Of all explanations considered, therefore, that of Neisser and Wechsberg seems to be the least likely. It would seem to us that Bordet's interpretation of these facts is borne out indirectly by certain experiments of Morgenroth and Sachs [46] themselves, in which the mutual quantitative relations between complement and "amboceptor" were studied. In these experiments it was shown that the more

[45] In this connection read also sections on zone phenomena in chapters on Alexin fixation and on precipitation and the section on the union of antigen with antibody.

[46] Morgenroth and Sachs. *Berl. klin. Woch.* No. 35, 1902.

highly cells were sensitized, the smaller was the quantity of complement which was needed for their hemolysis, and vice versa, the less the sensitization (the smaller the quantity of amboceptor) the more complement was necessary to produce the same result. The following extract from one of their protocols will illustrate this:

BEEF BLOOD CELLS 5%, 1 C. C., ANTIBEEF GOAT SERUM, GUINEA-PIG COMPLEMENT

Amount of amboceptor	Relative amount of amboceptor	Amount of complement for complete hemolysis
.05	1	.008
.2	4	.0025
.4	8	.0014

A similar relation may be observed by all who have occasion to work with hemolytic reactions. In the present connection this seems to bear out Bordet's interpretation, since, knowing the differences in functional efficiency of various complements for different hemolytic and bactericidal complexes, we could well expect that insufficient sensitization of a red cell or bacterial antigen, not particularly amenable to the complement employed, might fail to absorb it completely out of the serum, thus giving a negative result which would simulate complete lack of affinity.

This research of Morgenroth and Sachs seems further of fundamental importance in its contradiction of the regularly progressive quantitative relations which strict adherence to the "amboceptor" idea would seem to impose.

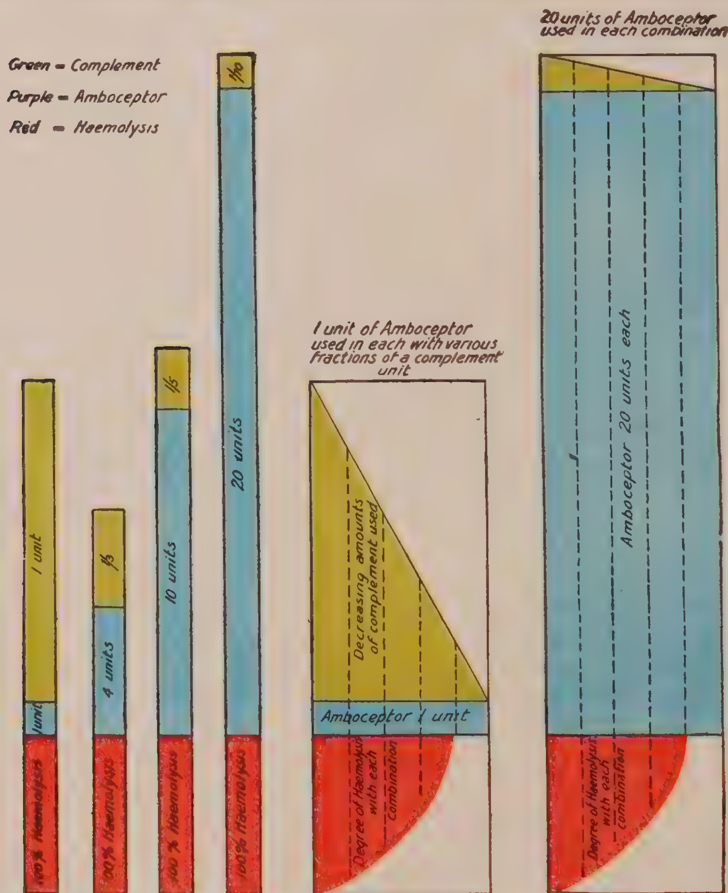
The quantitative relations here outlined have been diagrammatically represented by Noguchi as follows:

The essential point of difference between the opinions of Ehrlich and Bordet concerning the processes of hemolysis and bacteriolysis lies, as we have seen, in the conception of the union of alexin or complement with amboceptor or sensitizer. Although Ehrlich and his followers admit that the union of complement with amboceptor does not *usually* occur unless the amboceptor has previously united with the antigen, they still maintain that this may occasionally take place in the case of special complexes in which the complement may directly unite with free amboceptor. This, we have seen, is the basis of the Neisser-Wechsberg conception of complement—"Ablenkung" or deviation, and of other ramifications of this theory. Bordet, on the other hand, consistently holds that alexin or complement is attached only by the complex antigen-sensitizer (antigen-amboceptor).

The Conglutinin Effect.—In the controversy which this difference aroused, an observation was reported by Ehrlich and Sachs,[47] which seemed to represent, as they themselves express it, an "Ex-

[47] Ehrlich and Sachs. *Berl. klin. Woch.*, No. 21, 1902.

perimentum Crucis" proving Ehrlich's contention of the intermediary function of the amboceptor in contrast to Bordet's "sensitization" idea. The facts, as they record them, are as follows: When fresh horse serum is added to guinea pig corpuscles, slight hemolysis results. When inactivated ox serum alone is added to such corpuscles,



NOGUCHI'S DIAGRAM ILLUSTRATING THE QUANTITATIVE RELATIONS BETWEEN ANTIGEN, AMBOCEPTOR AND COMPLEMENT

(Taken from Noguchi, "Serum Diagnosis of Syphilis," Lippincott, Philadelphia, 1910.)

of course no hemolysis results. If the corpuscles are, on the other hand, exposed to the action of the inactive ox serum, together with fresh horse serum, very active hemolysis is brought about. Apparently the ox serum sensitizes (or furnishes amboceptor to) the guinea pig corpuscles, rendering them amenable to the action of the complement in the fresh horse serum. In other words, inactivated ox serum can be reactivated by the addition of fresh horse

serum. From this one would expect that if the guinea pig cells were exposed to inactive ox serum, then separated from the serum by centrifugalization and fresh horse serum subsequently added, hemolysis would ensue. However, this was not the case. When the cells were so treated it was found that they had not been sensitized, and, what is more, it could be shown that the ox serum so employed had lost none of its ability to produce strong hemolysis when added to another complex of cells and fresh horse serum. Ehrlich and Sachs concluded that this experiment definitely showed the ability of the amboceptor in the ox serum to unite with alexin independently. The relation to the cell occurred only after the union of the amboceptor in the ox serum and the complement in the horse serum had been established, and if their interpretation is correct, of course, it constitutes strong evidence against the general principle of "sensitization" as conceived by Bordet.

This apparent inability of the corpuscles to absorb amboceptor independently out of the inactivated ox serum, and the fact that hemolysis results only if the corpuscles, ox serum, and fresh horse serum are all simultaneously present, are extraordinary and not at all in keeping with the preceding work of Ehrlich and Morgenroth, and indeed with experience of these phenomena in general. It is logical therefore to examine more closely the peculiar conditions maintained in these experiments before applying the reasoning deduced from obviously different phenomena to their explanation.

Bordet and Gay [48] accordingly studied the Ehrlich-Sachs phenomenon carefully and obtained results which confirmed the experimental data of these writers but cast much doubt upon the validity of their conclusions.

In going over the experiments of Ehrlich and Sachs, Bordet and Gay made an observation which had apparently escaped the attention of the former investigators. Heated bovine serum has but a slight agglutinating power for guinea pig corpuscles. Fresh horse serum agglutinates them only slightly and slowly. On the other hand a mixture of the two sera agglutinates them very rapidly and completely. The bovine serum apparently possessed an accelerating or fortifying influence both upon the weakly active normal hemolysins and agglutinins in the horse serum. Bordet and Gay consequently suspected that this property might be due to an undescribed substance, peculiar to the bovine serum. To eliminate the uncertain elements obtaining in experiments in which normal sensitizer is used they now experimented with guinea pig corpuscles, anti-guinea pig sensitizer (from a rabbit immunized with guinea pig blood cells) and guinea pig alexin.

They found that sensitized guinea pig cells are hemolyzed by guinea pig alexin very slowly and imperfectly, as is often the case

[48] Bordet and Gay. *Ann. de l'Inst. Past.*, Vol. 20, 1906, p. 467.

when the alexin comes from the same animal species as the cells. When heated bovine serum was added to the complex of sensitized cells and alexin, rapid agglutination and hemolysis resulted. Their experiments may be tabulated as follows:

1. Cells + guinea pig alexin + heated bovine serum = no agglutination; very slight hemolysis on next day.
2. Cells + sensitizer + heated bovine serum = slight agglutination; no hemolysis.
3. Cells + sensitizer + alexin + bovine serum = powerful agglutination and complete hemolysis in 10 minutes.
4. Cells + sensitizer + alexin = very slight agglutination and incomplete hemolysis in 30 minutes.
5. Cells + sensitizer = slight agglutination; no hemolysis.

In tube (1) the slight hemolysis was due to the small amount of normal sensitizer present in the bovine serum, and the slight agglutination in tube (5) is referable to the agglutinating power of the sensitizer. In tube (3) we see the powerfully accelerating effects exerted both upon agglutination and hemolysis when bovine serum acts upon sensitized corpuscles in the presence of alexin.

Bordet and Gay's interpretation of the Ehrlich-Sachs phenomenon, in the light of these new experiments then, is, in their own words, as follows: "When guinea pig corpuscles are added to a mixture of the two sera they are affected by the sensitizer of the horse serum and, to a certain extent, by the sensitizer in the heated bovine serum. This second sensitizer is, however, superfluous. Its presence is by no means necessary for the experiment. When this sensitization is effected the corpuscles are then in condition to fix the horse alexin. This alexin, however, has only slight hemolytic power. But once the corpuscles have become sensitized and laden with alexin they are modified in their properties of molecular adhesion to such an extent that they become able to attract a colloidal substance of bovine serum, which unites with them. The adhesion of this new substance produces two results: it causes the blood corpuscles to be more easily destroyed by alexin and also agglutinates them energetically. Consequently, a powerful clumping, followed by hemolysis, is observed."

Bordet and Gay, therefore, assume that the action of the bovine serum is due to a new substance which they speak of as "bovine colloid." This substance resists heating to 56° C., is probably albuminous, and has the property of uniting with cells that are laden with sensitizer and alexin, but remains free in the presence of normal or merely sensitized cells.

They fortify this opinion by showing experimentally that the "colloid" is removed from bovine serum by absorption with sensitized bovine corpuscles which have been treated with horse alexin.[49]

[49] Browning (*Wien. klin. Woch.*, 1906) had shown that horse alexin may be absorbed by sensitized beef cells without causing hemolysis.

Bordet and Streng [50] later studied this "colloid" more thoroughly and have suggested for it the name *"conglutinin."* Streng [51] later showed that the agglutinating action of this substance could be shown not only for sensitized and "alexinized" red blood cells, but also for similarly treated bacteria, and that conglutinins were present not only in bovine serum, but in that of goats, sheep, antelopes, and a number of other herbivores, but apparently absent in cats, dogs, guinea pigs, and birds.

The body described by these workers as conglutinin is probably identical with a similar heat-stable serum component reported by Manwaring [52] and called by him "auxilysin."

[50] Bordet and Streng. *Centralbl. f. Bakt.*, Orig. Vol. 49, 1909.
[51] Streng. *Zeitschr. f. Immunitätsforsch.*, Orig. Vol. 2, 1909, p. 415.
[52] Manwaring. *Centralbl. f. Bakt.*, 1906; Orig. Vol. 42.

CHAPTER VII

FURTHER DEVELOPMENT OF OUR KNOWLEDGE CONCERNING COMPLEMENT OR ALEXIN. COMPLEMENT FIXATION

Origin of Alexin in Leukocytes.—It will be remembered that Buchner in his first studies upon the "alexin" compared its action to that of an enzyme or ferment, and suggested that the source of this substance might possibly be found in the white blood cells. This thought was very obviously suggested by the observation that bacteria were destroyed within the white blood cells, after phagocytosis, by a process analogous in many ways to that by which they were destroyed by the serum constituents. Hankin,[1] in an elaborate study dealing with the problem, maintained the leukocytic origin of alexin on the basis of the observation that increased bactericidal properties closely followed upon the heels of periods of leukocytosis. He assigned the particular property of alexin production to the eosinophile cells, proposing for them the designation "alexocytes." Further study, however, has not justified such an association with the eosinophiles, and Hankin's opinion has not been experimentally upheld.

After Hankin the problem occupied the attention of a number of other investigators, and many of them succeeded in showing that there was, indeed, an increased bactericidal power in exudates rich in leukocytes, and further that bactericidal substances could be directly extracted from leukocytic emulsions. We refer particularly to the early work of Denys and Havet,[2] of Hahn,[3] of Van de Velde,[4] and others, studies which will be described in our chapter on phagocytosis. This work was done before the complex nature of the bactericidal constituents of serum had been demonstrated and before the work of Schattenfroh and others had shown that the bactericidal substances extracted from leukocytes were of a nature quite distinct from the active elements of the serum, and were independent of the participation of alexin. Although these earlier investigations cannot properly be regarded, therefore, as proving the leukocytic origin of

[1] Hankin. *Centralbl. f. Bakt.*, Vol. 12, 1892.
[2] Denys and Havet. *La Cellule*, Vol. 10, 1894.
[3] Hahn. *Archiv f. Hyg.*, Vol. 25, 1895.
[4] Van de Velde. *La Cellule*, Vol. 10, 1894.

alexin, Metchnikoff and his school have nevertheless adhered to this conception for various additional reasons.

Metchnikoff distinguishes between two kinds of alexin—the *microcytase,* which is the bactericidal complement or alexin, and is supposed to originate from the microphages or polynuclear leukocytes, and the *macrocytase,* which represents the hemolytic and cytolytic alexin or complement, and originates from the mononuclear cells or macrophages. As in the case of the bactericidal alexin, extraction methods have been employed to demonstrate that the hemolytic alexin took its origin in the macrophages, and at Metchnikoff's suggestion, Tarassewitch [5] prepared hemolytic substances by extracting spleen tissue and other "macrophagic organs" in various ways. Here again the identity of the hemolytic extracts with serum hemolysins has been placed in doubt. Korschun and Morgenroth [6] have shown that the hemolytic organ extracts were heat stable and alcohol soluble; Donath and Landsteiner,[7] and others, have obtained similar results. It would be quite thankless to review the extensive literature which has accumulated upon this point. It would seem, in summarizing it, that no definite proof of the presence of true, active alexin, either hemolytic or bactericidal, within the leukocyte or mononuclear cells has been brought by methods of extraction, and the apparently positive results reported by earlier observers are adequately explained by the discovery of the heat-stable and non-reactivable bactericidal and hemolytic substances in extracts of such cells by Schattenfroh, Korschun and Morgenroth, and many others. It appears, moreover, from these investigations that probably the intracellular substances by which the digestion of ingested bacteria or blood cells is brought about are of a nature entirely distinct from that of the serum antibodies and alexins. A very ingenious demonstration of this is found in an experiment first made by Neufeld. Neufeld [8] allowed leukocytes to take up highly sensitized red cells. Instead of undergoing prompt hemolysis, as they would if small amounts of alexin had been added, they were slowly broken up without hemolysis, fragments of hemoglobin remaining after complete morphological disintegration of the erythrocytes. At no time were intraphagocytic "shadow" forms observed.

The failure to extract alexins from dead leukocytes does not, however, preclude the possibility of the secretion of alexins by living leukocytes. This point is one which is, of course, much more difficult to investigate directly. Indirectly the increased bactericidal properties of exudates rich in leukocytes, as found by Denys and Havet, would point in this direction. However, even this is not

[5] Tarassewitch. Cited from Metchnikoff.
[6] Korschun and Morgenroth. *Berl. klin. Woch.,* No. 37, 1902.
[7] Donath and Landsteiner. *Wien. klin. Rundschau,* Vol. 40, 1902.
[8] Neufeld. *Arb. a. d. kais. Gesundheitsamt,* Vol. 28, 1908, p. 125.

conclusive, since at the time when these investigations were carried out no discrimination was made between the bactericidal serum substances and those other "endolysins" which might well have been extracted from the accumulated white blood cells. The writer some years ago attempted to approach this problem directly by keeping leukocytes alive in inactivated serum and in Ringer's solution at 37.5° C. for several days in the hope that, after 48 hours, alexin, hemolytic or bactericidal, might appear in these fluids. The experiments were entirely negative, but were regarded as inconclusive, since it was impossible to determine accurately how long, or in what proportion, the leukocytes had remained alive.

One of the basic premises of Metchnikoff's theory on the nature of alexin consists in the conception that alexin is not found in the circulating blood plasma, but appears only when there has been leukocytic injury, as in the clotting of blood or in the "phagolysis" which, as we have seen in the chapter on phagocytosis, usually occurs after foreign substances have been injected into the peritoneum, preceding a local accumulation of leukocytes. This point of view seems to be rendered improbable because of the rapid hemolysis which occurs when we inject sensitized red blood cells into the circulation of an animal, but we might here, too, assume a preliminary injury to white blood cells resulting from the intravenous injection of foreign material.

Much less likely to be accompanied by cell injury is the method of obtaining blood serum by creating an area of artificial edema by ligating a limb—or, as in Metchnikoff's [9] experiments, the ear of a rabbit. And, indeed, in edema fluids so obtained little or no alexin is ordinarily found. This fact has been interpreted in favor of Metchnikoff's views, as has also the curious absence of alexin in the aqueous humor of the anterior chamber of the eye.[10] In this fluid no alexin is present under normal conditions, but if puncture is practiced, and the fluid again taken after a period of three or four hours, alexin is now found, probably, according to Metchnikoff's school, because of the coincident entrance of leukocytes into this space. It is conceivable, however, that the aqueous humor may be free from alexin for other reasons than the absence of leukocytes; and an injury which is followed by the invasion of leukocytes is pretty sure to be followed also by the entrance of the fluid elements of the blood; i.e., alexin.

Is there Alexin in the Circulating Blood?—Much experimental work has been done in which it has been attempted to demonstrate directly that the blood plasma contains no complement or alexin. The most important investigation of this kind is that carried out

[9] Metchnikoff. *Ann. de l'Inst. Past.*, Vol. 9, 1895. Bordet. *Ann. de l'Inst. Past.*, Vol. 9, 1895.

[10] Metchnikoff. *Loc. cit.* Mesnil. *Ann. de l'Inst. Past.*, Vol. 10.

by Gengou [11] in 1901. It was Gengou's primary purpose to obtain the plasma of mammals in such a way that no cell injury would occur. This he accomplished by special methods in which coagulation was avoided without the addition of foreign anticoagulants like hirudin, etc. His technique was, in essence, as follows: He took the blood directly through a paraffined cannula into tubes that had been coated with paraffin, and centrifugalized it at low temperatures until cell free. This plasma, taken from the paraffin tubes, quickly clotted, and the material with which the experiments were done actually consisted of blood serum. Upon examining the serum so obtained, he found that it exerted practically no bactericidal action. As a result of this investigation he claims to have demonstrated the truth of Metchnikoff's contention that the circulating blood plasma contains no alexin.

If borne out, it is true that Gengou's results would very powerfully support this theory, and for this reason a large number of experiments have been made since then, with the same end in view.

In all such investigations the technical procedures are extremely difficult and, as Addis [12] has recently said, in our opinion quite correctly, it would be impossible to carry out bacteriolytic or hemolytic experiments with mammalian paraffin plasma without obtaining coagulation, and for this reason most of the writers who have repeated Gengou's experiments have worked, as he did, not with plasma, but with serum. Falloise,[13] following Gengou's method exactly, obtained results diametrically opposed to those of Gengou; Schneider,[14] also with the same technique, failed to confirm Gengou's results; Herman,[15] on the other hand, confirms Gengou.

In order to overcome the technical difficulties encountered in working with mammalian plasma a number of writers have more recently experimented with bird blood, which, as is well known, coagulates much less easily than does mammalian blood. Hewlett,[16] who worked with goose plasma and peptone plasma, could not confirm Gengou's results. Lambotte,[17] examining the plasma of chickens, found no difference between the serum and plasma in their contents of bactericidal alexin, as measured against cholera spirilla. Von Dungern, working with fish plasma, obtained similarly negative results, and recently Addis, in a careful comparative study of chicken plasma, found no evidence of differences between plasma and serum in either the bactericidal or the hemolytic alexin. As far as we can tell at present, therefore, we cannot accept, as conclusively

[11] Gengou. *Ann. de l'Inst. Past.*, Vol. 15, 1901.
[12] Addis. *Jour. Inf. Dis.*, Vol. 10, 1912.
[13] Falloise. *Bull. de l'Acad. Roy. de Méd.*, 1905, p. 230.
[14] Schneider. *Archiv f. Hyg.*, 1908, Vol. 65, p. 305.
[15] Herman. *Bull. de l'Acad. Roy. de Méd.*, 1904, p. 157.
[16] Hewlett. *Archiv f. exp. Path. u. Pharmk.*, 1903, Vol. 49, p. 307.
[17] Lambotte. *Centralbl. f. Bakt.*, I, Orig., 1903, Vol. 34, p. 453.

proven, the contention that the circulating plasma contains no alexin. Nevertheless the Metchnikoff school have not been discouraged by the various contradictions of Gengou's work, found in the experiments we have enumerated, because they are not satisfied that the technique of other workers has conclusively excluded cell injury. Owing to the great difficulties of investigations of this kind, when carried out with mammalian blood, it is not impossible that they are justified in this, but nevertheless the assumption of the absence of alexin in the plasma finds so many objections in other observations that the burden of proof would certain rest with Gengou and his supporters. Not the least important of these objections, it seems to us, is based on the very simple experiment of injecting bacteria into the veins of a living animal and finding a very rapid and active phagocytosis. And considering the very probable participation of alexin in the opsonic functions this would seem to point strongly toward the presence of these substances in the circulating blood. The evidence also furnished by the recent developments of our understanding of anaphylaxis would further tend to strengthen our belief in the presence of alexin or complement in the normal circulation. For, in the process, as we shall see in a later chapter, complement plays an important rôle. When 3 per cent. salt solution is administered (as in Friedberger's experiments), and the action of complement is thereby inhibited, anaphylactic shock may be greatly diminished.

It has also been claimed, chiefly by Walker [18] and by Henderson Smith,[19] that, as serum stands upon the clot it at first gains in alexin or complement contents, an occurrence which they attribute to the liberation of alexin from the leukocytes. This observation has not been universally borne out and, even were it unquestionable, it might be dependent upon any one of the numerous factors involved in the complicated process of coagulation rather than upon leukocytic changes only.

Alexin and Glandular Organs.—The failure to obtain definite proof of the origin of alexin from the white blood cells has led to search for the source of these substances in various organs. An interesting series of investigations on this subject are those of Mlle. Louise Fassin,[20] who believes that she has found reasons for definitely associating the thyroid gland with alexin production. She found that the subcutaneous injection of thyroid extract into dogs and rabbits was followed by a rapid increase of alexin, both hemolytic and bactericidal, and that the same thing was true when thyroid substance was administered by mouth. When the thyroid gland was removed from rabbits a reduction of alexin resulted. Although important, these researches do not necessarily prove that

[18] Walker. *Jour. Hyg.*, Vol. 3, 1903.
[19] Smith. *Proc. Roy. Soc.*, Series B, Vol. 79, 1906.
[20] Louise Fassin. *C. R. de Soc. Biol.*, Vol. 62, 1907.

the thyroid can be looked upon as a source of alexin, and, indeed, Fassin gives experimental results without drawing any very sweeping conclusions. It might well be that the thyroid secretion is simply concerned in stimulating the production of alexin from another source. Marbé [21] has similarly associated the thyroid gland with the production of opsonins, which, when we consider the probable identity of alexin and normal opsonin, may be taken as a confirmation of Fassin's work.

Of great interest also are the series of investigations which associate the liver with the production of alexin. The basis of such investigations is found in the observations made by Morgenroth and Ehrlich [22] that there is a diminished production of complement or alexin in dogs subjected to phosphorus poisoning, with consequent degeneration of the liver. The first investigator to study this question experimentally was Nolf.[23] Nolf tried to approach it by extirpating the liver in dogs, and found that his results were unreliable by this method. He then experimented with rabbits and found that when the liver was extirpated in these animals and the vena cava anastomosed with the portal vein (Eck fistula) the animals would survive for three or four hours. This period, though short, was sufficient to show definite changes in the blood. Taken just before death it differed from that taken just before the operation in a number of important respects. There was relative incoagulability, there was autohemolysis, and with these there occurred an extreme fall of alexin or complement. Serious objections may be brought against Nolf's experiments. In the first place the operation as performed by him results in shock and injury so profound that rapid death ensues, conditions under which not only the complement-producing functions but all functions, secretory and otherwise, are reduced. Müller [24] objects to Nolf's experiments chiefly for the reason that he did not prevent the absorption of toxic substances from the intestine, materials which could now enter the general circulation without any longer being neutralized by the liver functions. Müller, for this reason, repeated Nolf's work but, by a complicated technique, temporarily shut off the intestinal circulation in addition to extirpation of the liver. He found, in agreement with Nolf, that exclusion of the liver from the circulation resulted in the prompt diminution of complement or alexin. In all such experiments, however, the very profound shock which necessarily occurs in the animals would seem to us to vitiate the results. Moreover, Liefmann [25] has repeated Müller's experiments without being able to obtain the same results. Not satisfied with these experiments, however, Liefmann experimented on frogs;

[21] Marbé. *C. R. de la Soc. Biol.*, Vols. 64, *et seq.*, 1908–1909.
[22] Morgenroth and Ehrlich. In Ehrlich's "Gesammelte Arb.," etc.
[23] Nolf. *Bull. de l'Acad. de Science de Belg.*, 1908.
[24] Müller. *Centralbl. f. Bakt.*, Vol. 57, 1911.
[25] Liefmann. *Weichhart's Jahresbericht*, Vol. 8, 1912, p. 155.

in whom, as Friedberger has shown, extirpation of the liver is not so rapidly fatal as in warm-blooded animals. He removed the livers of frogs in a number of cases and, although his animals lived about a week, there was no definite diminution of the hemolytic properties of the serum. It seems, therefore, that the origin of alexin in the body is by no means settled and requires a considerable amount of further investigation.

Nature of Alexin.—Equally unsatisfactory have been the attempts to define the chemical nature of the complement or alexin. In the investigations dealing with the hemolytic action of cobra venom it seemed at first as though a clue to this problem had been found. Flexner and Noguchi [26] made the interesting observation that cobra poison alone does not hemolyze the blood cells of certain animals, namely those of cattle, goats, or sheep, if these cells are washed entirely free of serum. This seemed to suggest that the serum of these animals contained some activating substance. It also seemed to indicate that the cells of other animals, which were easily hemolyzed, even when entirely freed of serum, might contain such an activating substance within themselves. The behavior of this activating substance toward snake-venom hemolysis was therefore very similar to the action of complement, except in one important respect, namely, as Calmette [27] showed, almost all sera were rendered more efficient for the activation of snake venom when heated to 65° C., whereas complementary properties of sera for other hemolyzing complexes are, of course, destroyed at 56° C. Kyes,[28] on further studying these phenomena, extracted the red blood cells of rabbits and other animals whose cells were hemolyzed by snake venom alone, by shaking them up with distilled water, and showed that, with these extracts, he could activate the venom against ox, goat, and sheep corpuscles, cells which were not ordinarily hemolyzed by the venom without the addition of serum. Similar activation of the venom with extracts of the ox, goat, or sheep corpuscles was not possible. He concluded from this that the blood cells of the rabbit, dog, guinea pig, and man possessed an "endocomplement" for the snake venom; that is, a complementary substance contained within the cells, while in the other species it was found in the activating serum only.

The thermostability of such venom "complements" encouraged him to attempt their isolation, and he found that they were ether-soluble, indicating their lipoidal nature; and, finally, after several negative attempts with activation by other lipoids, he determined that lecithin, added to the corpuscles and the snake venom, brought

[26] Flexner and Noguchi. *Jour. Exp. Med.*, Vol. 6, 1902; *Univ. Pa. Med. Bull.*, 1902 and 1903.

[27] Calmette. *C. R. de l'Acad. des Sciences*, p. 134, 1902.

[28] Kyes. *Berl. klin. Woch.*, Nos. 38 and 39, 1902. Kyes and Sachs. *Berl. klin. Woch.*, Nos. 2-4, 1903.

about a rapid hemolysis. This seemed to explain both why heated serum could activate the venom in some cases, and why some varieties of blood cells could be hemolyzed without serum, since lecithin is a substance widely distributed both in the fluids and cells of the animal body. His further studies seemed to show that, by proper chemical manipulation (bringing together cobra poison with lecithin in chloroform solution), he could produce a combination of the two which he called "cobra lecithid," a substance which apparently "activated" cobra venom. He conceived it as the "amboceptor-complement" complex of the cobra hemolysin, which acted hemolytically upon all varieties of blood cells.

These researches of Kyes aroused much interest, chiefly because they seemed to furnish an example of a chemically definable complement, lipoidal in its constitution. Recent researches by Von Dungern and Coca,[29] however, seem to prove that, while Kyes' experimental facts were perfectly accurate, his conclusions do not seem to have been warranted. Von Dungern and Coca showed that the cobra venom contains a lipoid-splitting ferment which acts upon the lecithin, liberating substances from it which hemolyze in the same way as do many other non-specific substances. The cobra-lecithid, according to this, would represent merely a lecithin derivative which happens to have hemolytic action without any specific relationship to the hemolytic properties of the venom itself. Thus, even in this case, unfortunately, we are not in possession of facts which bring us nearer to a chemical understanding of the complementary substances of alexins.

In the further development of attempts to define alexin or complement chemically, two further researches are of importance, namely, those of Von Liebermann [30] and of Noguchi. In both investigations it is suggested that the alexin may consist of a combination of soaps and proteins. Noguchi [31] showed that the hemolytic organ-extracts described by various observers were soaps, a possibility which had been previously considered by Sachs and Kyes.[32] Noguchi further established analogies between his soaps and complement as follows: Sensitized blood cells are hemolyzed by mixtures of soaps and inactivated guinea pig serum, while normal erythrocytes are not hemolyzed by similar mixtures; furthermore, like normal complement, such serum-soap mixtures are inactivated by prolonged preservation and by heating at 56° C. Objections were soon made to the findings of both Noguchi and Von Liebermann by Hecker,[33] whose experiments seemed to show that when sensitized blood cells were thoroughly washed free of serum soaps did not have

[29] Von Dungern and Coca. *Münch. med. Woch.*, 1907, p. 2317.
[30] Von Liebermann. *Biochem. Zeitschr.*, Vol. 4, 1907.
[31] Noguchi. *Biochem. Zeitschr.*, Vol. 6, 1907.
[32] Sachs and Kyes. *Berl. klin. Woch.*, 2-4, 1903.
[33] Hecker. *Arb. a. d. königl. Inst. f. exp. Ther.*, Heft 3, 1907.

this hemolyzing action, and Friedemann and Sachs [34] claimed that they were unable in any case to inactivate the hemolytic serum soap mixtures by heating to 56° C. These writers, as well as others, attribute Noguchi's results to the fact that the sera which he used to produce his "artificial complement," i. e., his serum soap mixtures, were heated to 50 to 51° C. only, a fact which would justify doubt of complete inactivation. Knaffl-Lenz [35] has more recently carried out experiments on the same question. His results seem to show that the hemolytic action exerted by fatty acids or soaps is a phenomenon quite incomparable to true complement action, and that these hemolysins are heat stable, remaining unchanged by heating at 56° C. We have referred in a number of places to the analogy between alexins and ferments or enzymes. The chief objection to this conception formerly brought forward was based upon the fact that the complement or alexin, unlike an enzyme, was used up during its reactions, and that a definite quantitative relationship existed between the alexin and the amount of cells or bacteria upon which it could act. Recent experiments by Kiss [36] seem to show that this quantitative relationship is not as strict and regular as was formerly supposed. He showed that the action of complement depends very largely upon its concentration. For instance, to cite his work directly: "0.05 complement is sufficient to hemolyze completely a definite quantity of sensitized blood cells if the experiment is done in a total volume of 5 c. c. 0.02 c. c. of complement gives absolutely no hemolysis in a similar volume. When, however, the total volume is reduced to 2.5 c. c., then 0.02 c. c. of the complement begins to act, and it produces complete hemolysis if the total volume is reduced to 1.25." In further developing this observation he showed that, if sufficiently concentrated, a very small amount of complement can act upon an extremely large amount of red blood cells, an amount incomparably larger than those acted upon in more dilute solutions. These observations would tend to strengthen considerably the conception of the ferment nature of alexins in general. Moreover, investigations, such as those of Northrup discussed in the Section dealing with the union of toxin and antitoxin, have considerably changed the older ideas in which it was assumed that enzymes were not used up or—more properly speaking—inhibited in the course of their action.

Kiss' observations are furthermore in agreement with the investigations of Liefmann and Cohn,[37] whose work we have mentioned in the preceding chapter on Cytolysis. These writers assert that the fixation of complement during hemolysis is not due to its chemical union with the sensitized cells, but is due to fixation by the end

[34] Friedemann and Sachs. *Biochem. Zeitschr.*, Vol. 12, 1908.
[35] Knaffl-Lenz. *Biochem. Zeitschr.*, Vol. 20, 1909.
[36] Kiss. *Zeitschr. f. Imm.*, Vol. 3, 1909.
[37] Liefmann and Cohn. *Zeitschr. f. Imm.*, Vol. 8, 1911.

products of the reaction; in other words, by the stromata of the red cells and possibly by other substances given up by these cells. A further factor contributing to the disappearance of complement in such reactions is, they claim, its rapid deterioration at 37° to 40° C., when diluted. If they are right, these considerations also remove important objections to the conception of complement as a ferment.

It is clear, therefore, that although we have gained much detailed information regarding the functional activity of the complement or alexin, and may assume, in a general way, that its action is similar to, if not identical with, that of an enzyme, we are nevertheless still very much in the dark concerning its chemical nature. The same thing may be said in regard to its physical characteristics. One method of investigating the physical properties of complement has been that of filtration. It may be remembered that one of Ehrlich and Morgenroth's [38] arguments in favor of the multiplicity of complement was the fact that, when goat serum was filtered through a Pukal candle, the complement which was active upon rabbit corpuscles was retained, while that which acted upon guinea-pig cells passed through. Immune bodies or amboceptor always passed through.

Vedder,[39] in similar experiments upon bactericidal complements, claims to have been able, in the same way, to separate the complements acting upon different bacteria. The problem has been more recently investigated by Muir and Browning.[40] Their conclusions are briefly as follows: In the early stages of filtration through a Berkefeldt filter complement is often completely held back. After continued filtration it begins to pass through. If the complement is inactivated by the addition of hypertonic salt solution (5 per cent.), it passes through, and the filtrate can be reactivated by dilution to isotonicity. Sensitizer or amboceptor always passes through. Just how these experiments are to be interpreted is a little obscure. The fact that the addition of salt renders the complement capable of passing through the filter would seem to indicate that its original inability to permeate did not depend upon the size of the molecule. On the other hand, it is also possible that the addition of salt to the complement may increase its dispersion in such a way that the individual particles are rendered smaller. This, however, is purely speculative, and we are at a loss for a fully satisfactory explanation of the results of Muir and Browning. We have repeated some of the experiments of Muir and Browning and, in substance, confirmed their results. It is our opinion that new filters remove complement by adsorption, just as this is accomplished when complement is shaken up with kaolin or other finely suspended material.

[38] Morgenroth and Ehrlich. Ehrlich's "Gesammelte Arbeiten," etc.
[39] Vedder. *Jour. Med. Res.*, Vol. 9, 1903.
[40] Muir and Browning. *Jour. Path. and Bact.*, Vol. 13, 1909.

Inactivation of Alexin by Heat.—While, of course, it is one of the basic attributes of alexin that 56° C. for one-half hour destroys it, the process by which this moderate amount of heating inactivates is not any more definitely understood than is the effect of similar or slightly greater degrees of heat upon toxins or enzymes. One of the earlier ideas was that possibly heating to these moderate temperatures, while not causing visible coagulation, nevertheless produced a condition of diminished dispersion, the proteins in which the complement was contained being in an entirely different state of colloidal equilibrium, with possible surface tension changes. As a matter of fact, a purely physical interpretation of inactivation was encouraged by the fact that other physical methods, such as prolonged shaking, allowing to stand without heat, and the addition of such things as neutral salts, could lead to definite, permanent, or temporary inactivation of the alexin. It is not unlikely, however, that it will be necessary to change these ideas in view of our more recent knowledge concerning the possibilities of chemical union of proteins with the ions of salts, of acids and of bases, and relationships between salt contents, on the one hand, and hydrogen ion concentration and temperatures at which inactivation occurs will have to be taken into consideration.

Effects of Salt Concentration on Alexin.—That the addition of salts of various kinds in quantities greater than isotonicity (or more than the equivalent of 0.85 = 0.9 per cent. NaCl)[41] exerts a profound action upon the activity of complement is well known. Nolf[42] noted this in 1900, and the problem has been studied since that time by many investigators. Von Lingelsheim,[43] who studied it in connection with his work on the refutation of the "osmotic" theories of immunity, showed that increasing the salt contents of serum (KNO_3, NaCl, K_2HPO_3, etc.) progressively diminished its bactericidal power. Hektoen and Ruediger[44] also, after a very thorough study of this phenomenon, conclude that the action of the salts in such cases is exerted upon the alexin or complement and not upon the heat-stable sensitizers, and that it probably depends upon "physicochemical" causes. However, the manner in which such salt-inactivation is brought about is, to a great extent, obscure. There is no visible precipitation from serum after the addition of salts sufficient in quantity to weaken its action. Nothing is, as far as we can tell, removed from solution, and yet there is temporary inactivation which, at the same time, renders the complement filtrable, facts from which we can only surmise some physical alteration.

[41] Alexin can be preserved in the refrigerator for long periods if hypertonic salt solution (15 to 25%) is added. It will again become active if isotonicity is restored with distilled water.

[42] Nolf. *Ann. Past.*, Vol. 14, 1900.

[43] V. Lingelsheim. *Zeitschr. f. Hyg.*, Vol. 37, 1901.

[44] Hektoen and Ruediger. *Jour. of Inf. Dis.*, Vol. 1, 1904.

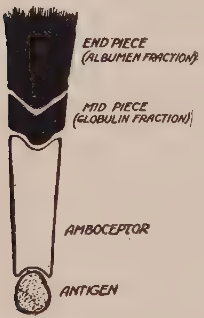
Inactivation of the complement also follows the removal of salts, but here the process is accompanied by a definite chemical change in that the serum globulins are precipitated.

Action of Acid and Alkali on Alexin.—It is well known that any considerable amount of acid or alkali added to fresh serum permanently removes the alexin function. Brooks [45] has determined that hydrogen ion concentrations high enough to transform serum proteins from the cation to the anion condition, that is, passed the iso-electric point, permanently inactivate it. Coulter [46] studied the activity of alexin at various hydrogen ion concentrations, both with and without the influence of heat. He took into consideration the complex structure of complement into albumin and globulin fractions. He found that the inactivation which complement undergoes when heated in distilled water dilutions is closely related to the properties of the euglobulin fraction, since the destruction is least at the reaction at which euglobulin is least soluble. The euglobulin sediment from serum washed in water and brought into solution again by the addition of NaOH at a Ph of 7.4, becomes least soluble on the addition of HCl, between Ph 5.1 and 5.7, and is iso-electric at about Ph 5. When examined in whole serum, however, the euglobulin has its iso-electric point at Ph 6.2 to 6.4, at which point Coulter found it existed not as a pure euglobulin, but as a compound of some other substance in the serum. At this reaction the destruction which complement undergoes on being heated, Coulter thinks depends on the relative preservation of the mid-piece function at this point. When salt is added, the destruction by heat increases as rapidly with the acidity as it does in the absence of salt, but on the alkalin side of this point the NaCl protects the destruction, according to Coulter, probably by a depression in the ionization of the euglobulin.

Alexin Splitting.—Studies of this process have led to important modifications in our conception of the nature of alexin, since they have shown that this body, formerly assumed to be single and homogeneous, may be subdivided into at least two component parts by a number of experimental procedures. Ferrata was the first one to point this out as a consequence of investigations undertaken by him primarily with the purpose of determining the nature of the influence of salts upon hemolytic processes. Older studies of Buchner and Orthenberger [47] had shown that bactericidal action was inhibited when salts were removed from the medium, but the causes underlying such inhibition had not been made clear. Ferrata [48] found, in the first place, that the absence of salts exerted no effect upon the

[45] Brooks. *Jour. Gen. Physiol.*, 3, 1921, 185.
[46] Coulter. *Jour. Gen. Physiol.*, 3, 1921, 771.
[47] Buchner and Orthenberger. *Archiv f. Hyg.*, Vol. 10, 1890.
[48] Ferrata. *Berl. klin. Woch.*, 1907, No. 13.

mechanism of sensitization, but that amboceptor or sensitizer became attached to the cellular elements as readily when salts were absent as when the reagents were suspended in normal salt solution. It was a natural inference, therefore, that the failure of hemolysis, which he observed in salt-free media (analogous to the similar experiences of Buchner in the case of bacteriolysis), must be attributed to failure of functionation on the part of the complement. On further investigation he obtained a very simple explanation. Ferrata removed the salts from his sera by dialyzing for twenty-four hours against distilled water. In this process, of course, there is a precipitation of the globulins while the water-soluble albumins remain in solution. The former may be redissolved in normal salt solution and the latter rendered isotonic by the addition of calculated amounts of concentrated salt. In this way the original serum components are divided into two parts, neither of which, as Ferrata found, is alone capable of producing hemolysis of sensitized cells. In order to obtain the complementary action possessed by the original serum it is necessary to combine the two. This principle discovered by Ferrata is probably responsible also for the results obtained by Sachs and Teruuchi,[49] who likewise noted the destruction of the complementary function in sera diluted with distilled water, but attributed this, in their publication, to the action of a complement-destroying ferment, which is assumed to be active in salt-free media only.



Conception of Complement - Splitting as First Suggested by Brand.

In his first experiments Ferrata reported that the precipitated globulin fraction was thermostable, the thermolability of complement being due entirely to the unprecipitated albumin fraction. The work of Ferrata was soon continued, however, by a number of other workers, who confirmed the essential fact of the partition of the complement but modified and considerably extended the original observations. Brand [50] found that both fractions were equally thermolabile, and that the globulin sediment, after being redissolved in salt solution, could not be preserved in an active condition for more than a few hours. Preserved in distilled water or as sediment, it may retain its activity for several days, but dissolved in salt solution it becomes inactive within 3 to 4 hours, at room temperature. Michaelis and Skwirsky [51] have since shown that the globulin fraction, thermolabile when free, is unaffected by a temperature of 56°

[49] Sachs and Teruuchi. *Berl. klin. Woch.*, 1907, Nos. 16, 17, and 19.
[50] Brand. *Berl. klin. Woch.*, 1907, No. 34.
[51] Michaelis and Skwirsky. *Zeitschr. f. Imm.*, Vol. 4, 1910.

C. after it has become attached to sensitized cells. Brand further studied the relationship of the two fractions to the sensitized cells and found that the globulin fraction may attach directly to such antigen-antibody complexes, but that the albumin fraction cannot be bound in this way unless the globulin fraction has been previously attached. For this reason he has referred to the former as the "end-piece" and the latter globulin sediment as the "mid-piece," assuming, on the basis of the conception of Ehrlich, that the globulin fraction serves to establish a link between the sensitized cell and the end-piece analogous to that formed by the "amboceptor" between the cell and the whole complement. It is possible, therefore, to treat sensitized cells with mid-piece in such a way that they are thereafter susceptible to hemolysis by the end-piece alone. Such cell-sensitizer-mid-piece combinations have been spoken of by Michaelis as *"persensitized"* cells.

Tsurusaki [52] confirmed the findings of Brand as to the thermolability of both "mid-piece" and "end-piece," but was unable to separate the complement of *normal* hemolysins into the two components in the same way, since he found that hemolytic power was, in such cases, completely destroyed after twenty-four hours of dialysis. It seems to us not impossible that the natural deterioration of alexic power which takes place during such periods of time, at temperatures of from 16° to 20° C., may easily be held accountable for this, since the very feeble sensitization of cells likely to take place in normal hemolysin complexes would require a correspondingly larger amount of alexin for activation.

We have mentioned that the so-called "mid-piece" undergoes a rapid change when dissolved in salt solution and, after 3 or 4 hours, may lose its ability to induce hemolysis when added to sensitized cells together with end-piece. Although Hecker [53] was able to confirm this, he nevertheless showed that this fact does not imply a destruction of the mid-piece. For when such apparently inactive "mid-piece" was added separately to sensitized cells, and end-piece was subsequently allowed to act upon the complex, hemolysis resulted. This seems to show that the "mid-piece" undergoes a change on standing in salt solution which does not alter its ability to combine with the sensitized cells, but which subjects it to inhibition of such union when end-piece is present. It is also a peculiar fact, evident in many of our own experiments, that when "mid-piece" and "end-piece" are first mixed and then added to sensitized cells the effect in hemolysis is less powerful than when the "mid-piece" is added to the cells first, and the "end-piece" later. This effect is so instantaneous that, if, in a series of experiments in which combina-

[52] Tsurusaki. *Biochem. Zeitschr.*, Vol. 10, 1908.

[53] Hecker. *Arb. a. d. könig. Inst. f. exp. Ther.*, Frankfurt a/M., Heft 3, 1907. See also Guggenheimer. *Zeitschr. f. Imm.*, Vol. 8, 1911.

tions of mid- and end-piece are used, the end-piece is run into the tubes containing the cells just before the mid-piece is added instead of the other way round, hemolysis is inhibited.

In working with dialysis, also, we have regularly had an experience which may explain the difficulties which many other investigators have had in such experiments. The globulin precipitate, which fell out after dialysis of 24 hours or more, almost without exception, retained moderate or slight hemolytic properties, which could not be removed until the precipitate had been dissolved in salt solution and reprecipitated with distilled water two or three times. This would imply that a minute amount of the end-piece, carried down in precipitation, must suffice to activate the mid-piece and would seem to point to the fact that in whole serum the two fractions are present as a complex and not separately. This question has been much discussed and many facts have been brought out on both sides. Hecker showed that the combination of mid-piece with the sensitized cells can take place at a temperature of 0° C., while that of end-piece with the "persensitized" cells requires a considerably higher temperature. The bearing this fact may have upon similar earlier experiments of Ehrlich and Morgenroth upon the thermal conditions governing the union of amboceptor and complement with antigen is self-evident. In the present connection, however, the fact that the two fractions may be separately absorbed out of the serum by sensitized cells at 0° C. would suggest the probability of their being separate in the whole blood. No crucial experiment has so far been possible, and there is not enough evidence on either side as yet to justify a definite opinion. However, the experiments of Michaelis and Skwirsky and later ones of Skwirsky alone have much indirect bearing on this question, though final interpretation is as yet impossible. Michaelis and Skwirsky,[54] after determining that an acid reaction inhibits the hemolysis of sensitized blood cells, found that under such conditions "mid-piece" alone is bound, but that "end-piece" or the albumin fraction is left unbound. They recommend the use of strongly sensitized cells in an acid medium as a method of obtaining free "end-piece" from serum.

Skwirsky [55] subsequently found that during the ordinary Wassermann reaction the complex of syphilitic serum and antigen binds the mid-piece only. If the Wassermann reaction has been strongly positive; that is if there has been absolutely no hemolysis, and we remove the supernatant fluid by centrifugation, active end-piece can be demonstrated in it by the addition of persensitized cells. Bronfenbrenner and Noguchi have also studied this phenomenon, but do not believe that Skwirsky's experiments prove that end-piece is free in such "fixation" supernatant fluids. These supernatant fluids, ac-

[54] Michaelis and Skwirsky. *Zeitschr. f. Imm.*, Vol. 4, 1910.
[55] Skwirsky. *Zeitschr. f. Imm.*, Vol. 5, 1910.

cording to them, differ from all other "end-pieces" in that they are active upon persensitized sheep corpuscles only, but not upon other cells. An explanation for this is lacking.

There is much that is confusing in the facts so far revealed about the two component parts of the alexin. The most difficult fact to explain is the peculiar inactivation of the mid-piece in salt solution, which prevents its functionation in the simultaneous presence of end-piece, but does not seem to interfere with its ability to combine with the sensitized cells. As was to be expected, explanation for this has been sought by the Ehrlich school in changes of affinity. Sachs suggests that the mid-piece, by its preservation in salt solution, has lost its avidity for the sensitized cells and has gained in avidity for the end-piece, an alteration which therefore prevents its union with the cells. The same idea was suggested by Hecker himself. It is a little difficult to reconcile this explanation, however, with the fact that whole serum can be preserved and remain active in its complementary function for a number of days, mid-piece and end-piece being present together, in a medium which, as far as salt contents are concerned, is isotonic with the salt solution in which mid-piece deteriorates so rapidly when alone.

That there is, after all, much similarity between the alexins of different animals is evident from the fact that, as Marks and other have shown, the end-piece of one animal may activate the mid-piece of another species. It appears also from experiments like those of Ritz and Sachs [56] that an animal may possess a mid-piece for certain sensitized cell complexes without possessing a corresponding end-piece. Thus they found that the serum of mice contained a mid-piece but not an end-piece active for the hemolysis of sensitized guinea-pig corpuscles.

Much that has been found out about the so-called globulin portion, moreover, tends to engender doubt as to the wisdom of applying to these complement fractions the terms "mid-piece" and "end-piece," an objection which is based upon reasons similar to those which prevent Bordet from accepting the term amboceptor. For so little is actually known concerning the mechanism of complement functionation, that it seems unwise to establish on a firm basis a preconceived idea of the mechanism by adapting the terminology to a theory. The most confusing feature of the problem lies in the surprising quantitative relations which seem to exist in the reactions of the two fractions. Thus Liefmann and Cohn [57] claim that in the presence of moderately sensitized cells no measurable amount of the so-called mid-piece or globulin fraction is bound, that is, removed from solution; and yet, when both fractions are added to such cells, rapid and complete hemolysis results. In the presence of heavily

[56] Ritz and Sachs. *Zeitschr. f. Imm.*, Vol. 14, 1912.
[57] Liefmann and Cohn. *Zeitschr. f. Imm.*, Vol. 7, 1910.

sensitized cells (20 to 50 units) a small quantity only is removed. Nevertheless this fraction has had a demonstrable effect on the cells, since it has rendered them amenable to the action of the albumin fraction. In all such experiments, therefore, as Liefmann justly points out, the degree of sensitization must be taken into consideration before conclusions are formulated. It is curious also that a slight excess of the globulin fraction may prevent complement action completely. In experiments cited by Marks [58] it appears that the most ineffective complement is obtained when "mid-piece" and "end-piece" are added to the sensitized cells in proportions of 1 to 1. If the proportion of "mid-piece" is increased two or threefold over that of "end-piece," hemolysis is inhibited. This, however, is true only when the two fractions are simultaneously added to the sensitized cells. When the sensitized cells are exposed to the excessive quantity of the "mid-piece" separately, and "end-piece" added later, the effect is one of stronger hemolysis than when smaller amounts are used. It is thus seen that the relations between the complement fractions in hemolysis are very involved. All that we can be sure of is that there are at least two separable parts, that one of these acts directly upon the sensitized cells, forming a so-called persensitized complex and rendering them amenable to the subsequent action of the unprecipitated albumin fraction.

The many difficulties encountered in the interpretation of the confusing phenomena observed in connection with this problem have, very naturally, led to a corresponding multiplicity of opinion. Most observers at present incline to the opinion that the globulin and albumin portions of fresh serum, separated by Ferrata's or any other of several common methods, represent actually two complement fractions. This is not, however, accepted by all workers. Bronfenbrenner and Noguchi [59] believe that the entire active complement is contained in the albumin fraction or so-called "end-piece." They hold that "complement-splitting" by dialysis or other methods is an inactivation of end-piece by change of reaction. In their experiments they were able to restore the functional activity of end-piece by the adjustment of reaction, either with acid or alkali, respectively, or by the addition of amphoteric substances. The mid-piece activates, they believe, by reason of its amphoteric nature and consequently adjusts any excessive acidity or alkalinity of the medium. They were able to substitute for mid-piece indifferent amphoteric substances such as alanin. Liefmann [60] has been unable to confirm the experiments of Bronfenbrenner and Noguchi, and believes that their results were caused by incomplete splitting of the complement. Incidental to a study of normal opsonins the writer has also repeated the experi-

[58] Marks. *Zeitschr. f. Imm.*, Vols. 8 and 11, 1911.
[59] Bronfenbrenner and Noguchi. *Jour. Exp. Med.*, Vol. 15, 1912.
[60] Liefmann. *Weichhardt's Jahresbericht*, Vol. 8, 1912.

ments of Bronfenbrenner and Noguchi without being able to confirm them.[61]

The method of Ferrata for the separation of the two parts of the complement is successful only if dialysis is very thorough and sufficiently prolonged to lead to complete precipitation of the globulins. Neufeld and Haendel[62] have had difficulty in thus separating the fractions, and the writer has noticed similar failures but has always been able to obtain eventual separation by sufficient prolongation of the dialysis. Because of the occasional difficulties and because of the time-consuming and inconvenient nature of the method other means of separation have been devised. The one used with success by many workers has been that introduced by Sachs and Altmann,[63] namely, precipitation of the sera with weak hydrochloric acid, $\frac{N}{300}$ to $\frac{N}{250}$. Liefmann has separated the components by precipitation of the globulins by the introduction of CO_2. In carrying out this method, Fraenkel[64] has found it advantageous to dilute the serum ten times with distilled water, then allowing the CO_2 to flow in at low temperatures. It is likely that any of the usual methods of globulin separation will serve for complement partition. The salting out methods are, however, extremely inconvenient because of the necessity for prolonged dialysis subsequently necessary to remove the salts.

Spontaneous Reactivation of Alexin.—The inactivation of complement or alexin by the addition of salts or by splitting is, very apparently, a temporary inactivation in which prompt restitution can be practiced by bringing back original conditions either by dilution to isotonicity or by reconstruction of the divided substance, respectively. Heating to 56° C., the simplest and most commonly employed method of inactivations was, until of late, regarded as an irreversible process, the complement being irretrievably destroyed in the procedure. Gramenitski[65] has recently carried out experiments which seem to show that this opinion is erroneous. His experiments were suggested by the fact, observed by Bach and Chodat,[66] that certain oxydases and diastases may spontaneously regain some of their activity after inactivation by heat. His work with complement indicated a similar gradual return to an active condition after moderate heating. The great theoretical importance of this observation will justify our insertion of one of Gramenitski's protocols.

Experiment 1. Complement 10 times diluted was heated to 56°

[61] Zinsser and Cary. *Jour. Exp. Med.,* Vol. 19, 1914.

[62] Neufeld and Haendel. *Arb. a. d. kais. Gesund.,* 1908.

[63] Sachs and Altmann. Cited from Sachs in "Kolle u. Wassermann Handbuch.," Vol. 2, p. 877.

[64] Fraenkel. *Zeitschr. f. Imm.,* I, Vol. 8, 1911.

[65] Gramenitski. *Biochem. Zeits.,* Vol. 38, 1912.

[66] Bach and Chodat. Cited from Gramenitski, *loc. cit.,* p. 511.

C. for 7 minutes. It was then tested against sensitized beef blood at varying intervals as follows:

Time after heating at which test was made	Quantity of hemoglobin gone into solution after			
	% 10 min.	% 20 min.	% 30 min.	% 40 min.
Immediately after heating.....	0	20	40	70
1½ hour................	0	30	60	80
24 hours................	20	70	80	100
48 hours................	10	40	70	....

In other experiments in which heating was more prolonged a similar regeneration was observed, though not as pronounced as in the one cited above. The largest amount of restored complement seemed to be present after about 24 hours. After this gradual deterioration again ensued. It is quite impossible to offer an adequate explanation for this at the present time. Gramenitski [67] acknowledges this, but permits himself certain speculations which we repeat in nearly his own language, since there is much in them which seems to us reasonable. The complement, as indeed all other active serum constituents, must be looked upon as colloidal in nature. When heat is applied to such substances alterations occur which gradually lead to coagulation. As this occurs there is an aggregation of particles and a consequent diminution of surface tension. This last point has been experimentally demonstrated by Traube,[68] who has regularly found a fall of surface tension as serum was heated to 56° C. And of greatest interest in this connection is the further determination by Traube that a gradual restoration of the surface tension takes place as the serum is allowed to stand. It is not inconceivable, therefore, that the inactivation of complement by heat may depend upon an alteration of its colloidal state, i. e., an aggregation of the particles, which, if not carried too far, may be reversible and followed by a gradual dispersion as the serum is kept 24 hours. On the same grounds the gradual deterioration of complement on standing may be compared to the slow settling out of colloidal suspensions which eventually results in spontaneous precipitation, a process which occurs not only in chemically well-defined colloids, but is often observed in sera. Bechold has referred to this as "das Altern Kolloidaler Lösungen."

Brooks has confirmed the observations of Gramenitski. He showed that after partial thermoinactivation, alexin recovers part of its hemolytic power, the recovery taking place more rapidly at 37° than at 7°. This recovery of power may restore at least one-third of its hemolytic action.

[67] Gramenitski. *Loc. cit.*, p. 504.

[68] Traube. *Zeitschr. f. Imm.*, Vol. 9, 1911, and *Biochem. Zeitschr.*, 1908.

Inactivation by Shaking.—Of great interest, furthermore, in connection with the physical properties of complement is the discovery made by Jacoby and Schütze[69] that complement can be inactivated by shaking. This astonishing observation has been confirmed by Zeissler,[70] Noguchi and Bronfenbrenner,[71] Ritz,[72] and others . It appears, according to these observers, that guinea-pig serum, when subjected to active shaking, can eventually be robbed thereby of its activating properties. The success of such experiments depends somewhat upon the concentration of the serum, and is best observed in a dilution of 1 part to 10 parts of salt solution. Under such conditions complete inactivation may be observed within 20 to 25 minutes. Between the inactivation of complement by heat and that which results from shaking, there are certain similarities which seem to strengthen the opinion regarding the nature of heat inactivation which we have cited above. For it has been variously shown that prolonged shaking of protein solutions, like heating, gradually leads to coagulation. It would be important to determine whether or not the inactivation by shaking, like that produced by heat, is accompanied by a fall of surface tension.

The Photoinactivation of Alexin.—There seems to be no question about the fact that the radiation of fresh serum with ultra-violet light to a slight extent inactivates alexin action. In this the temperature at which the radiation is done plays an important rôle. Brooks[73] particularly has studied this. By a very delicate method of alexin titration, he determined the actual inactivation by light radiation without possibility of doubt. The explanations for this which he gives cannot be gone into here since they involve complex considerations of photo-chemical reactions which we are not competent to appraise. We refer the reader to Brooks' original papers, the references of which are given.

ALEXIN OR COMPLEMENT FIXATION

The controversy regarding the multiplicity of alexin and the existence of a "complementophile group" cannot, of course, be regarded as closed, however much we may lean toward the acceptation of Bordet's point of view, since German experimenters of eminence still adhere to the Ehrlich interpretations. Moreover, it is, of course, extremely difficult to disprove such an assumption as that of the "polyceptor" conception of the complementophile group. However,

69 Jacoby and Schütze. *Zeitschr. f. Imm.*, Vol. 4, 1910.

70 Zeissler. *Berl. klin. Woch.*, No. 52, 1909.

71 Noguchi and Bronfenbrenner. *Jour. of Exp. Med.*, Vol. 13, 1911.

72 Ritz. *Zeitschr. f. Imm.*, Vol. 15, 1912.

73 Brooks. *Jour. Med. Res.*, 41, 1919, 411, and *Jour. Gen. Physiol.*, 3, 1921, 169 & 185.

we may safely assert that the functional unity of complement (and, after all, that is all that Bordet has maintained) is being upheld by the constantly increasing evidence in its favor which is being furnished by the practical and experimental application of the phenomenon of "alexin fixation" described, in 1901, by Bordet and Gengou.[74] It will be well to bear in mind that this phenomenon should be strictly distinguished from the so-called "complement deviation" ("Ablenkung"), described by Neisser and Wechsberg. The latter was advanced as an explanation of the inactivity of bactericidal sera when used in too great concentration, as described in another place (p. 176) (Neisser and Wechsberg phenomenon), and has been variously utilized as support for the assertion that alexin can unite with unattached sensitizer. It is regarded by most observers, moreover, as untenable in the light of later investigation. In spite of this, the term "Komplement-Ablenkung" has been employed by a number of German writers (see Citron, Vol. 2, "Kraus und Levaditi Handbuch") as synonymous with "fixation" in the sense of Bordet and Gengou.

The phenomenon of Bordet and Gengou, briefly described, is nothing more than an experimental utilization of the fact which we have discussed at length, that alexin is fixed by antigen and antibody after union, but by neither alone.

The condition, as observed by them, may be best described by submitting the protocol of the first experiment detailed in their communication:

An emulsion of a 24-hour slant of plague bacilli was used as antigen, heated antiplague horse serum represented the antibody, and fresh guinea-pig serum was used as alexin. A series of tubes was then prepared as follows:

1. Alexin + plague bacilli + inactivated antiplague serum.
2. Alexin + plague bacilli + inactivated normal horse serum.
3. Alexin + inactivated antiplague serum.
4. Alexin + inactivated normal horse serum.
5. Plague bacilli + inactivated antiplague serum.
6. Plague bacilli and normal horse serum.

These mixtures were left together for 5 hours and, at the end of this time, sensitized rabbit corpuscles were added to each tube. The result showed hemolysis in all the tubes except "1," in which there were plague bacilli, antiplague serum, and alexin, and in tubes 5 and 6, which had contained no alexin from the beginning.[75]

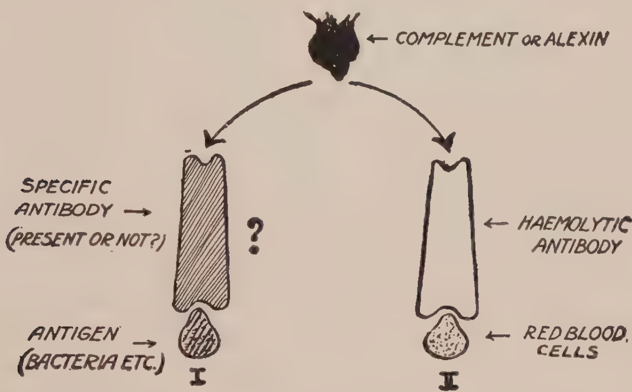
It was plain, therefore, that the bacilli when specifically sensitized had become capable of absorbing alexin and preventing its sub-

[74] Bordet and Gengou. *Ann. de l'Inst. Past.*, 1901, Vol. 15, p. 289.

[75] We will see later that unsensitized bacteria in emulsion will non-specifically fix small amounts of complement.

sequent action upon the sensitized erythrocytes. That the occurrence was not exceptional was shown by the fact that, in the same series, similar results were obtained with anthrax, typhoid, and proteus bacilli, and their respective antisera.

Schematized in accordance with the conceptions of Ehrlich our diagram would be as follows:



Complement Fixation Schematized According to Ehrlich's Views.

If the antibody in I is present then complement is fixed by the antigen-antibody complex, and is no longer free to act upon the hemolytic complex II. In the same way antigen I could be determined if a known antibody I were used. For, in the absence of either of these parts of the complex I complement would remain unfixed and free to act on complex II.

We represent the phenomenon graphically in the symbols of Ehrlich merely because they facilitate clearness of exposition.

In the presence of both parts of Complex I the alexin is held and is no longer available for Complex II. If either of the reacting parts, antigen or sensitizer, of Complex I are lacking the alexin is left unfixed and free to react with Complex II.

With this technique Bordet and Gengou were able to demonstrate, by indirect experiment, the presence of specific sensitizers in the sera of animals immunized with various bacteria, a fact which was, of course, surmised but had been amenable to proof heretofore only in the case of bacteria like the spirillum of cholera in which lysis under the influence of immune serum and alexin could be directly observed under the microscope. The practical possibilities of their method were, of course, immediately apparent. By the use of a known antigen specific sensitizers can be demonstrated in this way, and, vice versa, in the presence of a known antibody, the method will serve to identify the nature of a doubtful antigen. Thus bacterial differentiation can be carried out by adding to the suspected bacteria, in emulsion, a small quantity of a known antiserum and alexin, and determining whether or not the alexin has become fixed.

And, conversely, Bordet and Gengou [76] have more recently utilized the method in support of their claim of the specific etiological importance of the bacillus isolated by them from whooping cough, by showing that the serum of children suffering from this disease formed a specific alexin-fixing complex when treated with the bacillus.

The phenomenon of Bordet and Gengou thus found rapid practical application in the diagnosis of a number of infectious diseases, and has, of course, attained great clinical importance in the diagnosis of syphilis in the form of the "Wassermann" reaction and its many modifications. Before discussing these practical features in greater detail, however, it will be useful to discuss more particularly the many important theoretical considerations which have followed in the train of the complement-fixation phenomena.

Alexin-fixation with Non-cellular Antigens.—A year after the publication of Bordet and Gengou's paper Gengou [77] made another fundamentally important observation by showing that complement or alexin fixation was not limited to the complexes of cellular antigens and their antibodies, but that the sera of animals immunized with dissolved proteins (animal sera, etc.), when brought together with their specific antigens, likewise formed combinations which fixed alexin. Thus egg-white or dog serum, brought together with "anti-egg-white" or "anti-dog" rabbit serum, respectively, strongly fixed alexin, whereas neither the antigenic substances nor the antisera exerted such fixation alone. The interpretation put upon this by Gengou was the following: "In sera obtained by injecting rabbits with large doses of cow's milk, etc., there are, in addition to the precipitins of Bordet and Tschistovitch, substances analogous to the sensitizers described by Bordet in bacteriolytic and hemolytic sera, and later found in the majority of antimicrobial sera." The important point in this interpretation is that Gengou conceived the existence of antiprotein sensitizers, in addition to the precipitins, formed as a response to immunization with amorphous protein. Moreschi [78] soon confirmed Gengou's experimental determinations, and Neisser and Sachs [79] took the further logical step of applying this knowledge to the determination of proteins for forensic purposes. This, too, we will further discuss when we speak of the practical features of these phenomena. It thus appears that the fixation of alexin is a generalized property of all mixtures in which an antigen is brought into contact with its specific antibody, whether the antigen is in the form of the whole bacterial or other cell, or in that of a dissolved protein, animal serum, or egg-white, etc.

[76] Bordet and Gengou. *Ann. de l'Inst. Past.*, 1906.
[77] Gengou. *Ann. de l'Inst. Past.*, 16, 1902.
[78] Moreschi. *Berl. klin. Woch.*, No. 37, 1905.
[79] Neisser and Sachs. *Berl. klin. Woch.*, No. 44, 1905.

The observation of Gengou, though for a time insufficiently valued, has had a profound influence upon the subsequent understanding of serum reactions. The fundamental importance of this work was not fully recognized until his studies had found logical continuation in the investigations of Gay [80] and in those of Moreschi.

Moreschi [81] studied the antihemolytic properties possessed by the serum of a rabbit which had been treated with normal goat serum. He found that such a serum had distinct anticomplementary powers when it was added to a hemolytic system of ox blood sensitizer (obtained against ox blood from rabbits), and goat complement. With such a hemolytic system, however, there was anticomplementary action only against goat complement and not against rabbit or guinea-pig complement. If, however, he used a hemolytic system in which the amboceptor or hemolytic sensitizer employed was one obtained from a goat, the serum was anticomplementary for all complements which were used. Moreschi concluded from this that the apparent anticomplementary action of the serum could not be interpreted as the action of a specific anticomplement in the sense of Ehrlich, but that it resulted from the reaction which took place as the consequence of union of the antibody in the anti-goat rabbit serum and goat protein, which was introduced into the tubes, in the first case with the complement, and in the second with the amboceptor. He proved his contention by obtaining similar universal anticomplementary action when he added a little normal goat serum to the tubes set up as above described. It is plain, therefore, that anticomplementary action can be explained in observed cases by the simple consideration of the phenomenon of Gengou. Similar findings were later recorded by Muir and Martin,[82] and it may well be doubted, as a result of these and other researches, whether we are at all justified in assuming the existence of anticomplements.

Fixation of Alexin by Precipitates.—The work of Gay, published independently in the same year as that of Moreschi, has, in a general way, the same significance, but Gay recognized the relation of the conditions observed by him to the precipitin reaction, a feature absent from both the original study of Gengou and the work of Moreschi. Gay noticed that an inactivated hemolytic immune serum, left for some time in contact with its specific cells, and then separated from them by centrifugation, would often possess anticomplementary or anti-alexic properties. He further noted that after such a serum had been freed from the cells by a short centrifugation, if it was again vigorously centrifugalized, a slight, cloudy sediment would appear

[80] Gay. *Centralbl. f. Bakt.* I Orig. Vol. 93, 1905, p. 603.
[81] Moreschi. *Berl. klin. Woch.*, 1905, No. 37, *ibid.*, No. 4, 1906.
[82] Muir and Martin. *Jour. Hyg.*, Vol. 6, 1906. See also Muir's "Studies on Immunity," Froude, London, 1909.

at the bottom of the tubes. If this sediment was removed the serum lost its alexin-fixing properties. He recognized that the precipitate formed in these tubes was a specific precipitate resulting from the union of a precipitinogen and its antibody. The reaction was due entirely to the fact that insufficient washing of the cells used in producing the hemolysin, gave rise to the formation of precipitin against the serum of the animal from which the cells had been taken, and subsequently insufficient washing of the cells of this same species employed in the tests furnished enough antigen to give a precipitin reaction in the tubes in which the inactivated hemolytic (and precipitating) serum was mixed with the cells. Subsequently, numerous investigations [83] have shown Gay's interpretation to be correct, and we may now accept it as a fact that precipitates formed by the union of specific antigen with its antibody possess the power of fixing alexin and that, in a general way, this fixation is proportionate in energy to the amount of precipitate which is formed.

Gay utilized his results primarily to contradict certain assertions of Pfeiffer and Friedberger concerning antibacteriolytic substances supposed to occur in normal sera. These authors had found that, if normal sera possessing no "antagonistic" properties in the first place were left in contact with certain bacteria, they acquired antibacteriolytic properties for these particular bacteria. Thus normal inactive rabbit serum, left in contact with typhoid bacilli, and again separated from the bacteria, now prevented the lysis of sensitized typhoid bacilli if tested by the intraperitoneal method spoken of as the Pfeiffer reaction. Sachs [84] applied these observations to analogous hemolytic reactions and obtained similar results. He found that, if normal, inactive rabbit serum was left in contact with sheep or guinea pig corpuscles, it acquired the property of preventing the hemolysis of these corpuscles if, later, it was brought together with them in the presence of specific hemolysin and alexin. Gay now showed by experiment that Sachs' method was referable to insufficient washing of the corpuscles. When, in the first contact, the rabbit serum was exposed to the sheep corpuscles, a certain amount of sheep serum adherent to the cells was carried over into the rabbit serum. This sheep antigen later reacted with the antisheep precipitin present in the hemolytic immune serum and, in this way, fixed alexin and prevented hemolysis.

It seems that the analysis of Gay is correct, and that Sachs' conclusion as well as those of Pfeiffer and Friedberger, by analogy, cannot be taken as demonstrating the existing of specific anticomplements or anti-amboceptors. Gay has further offered the same mechanism as an explanation of the Neisser-Wechsberg phenomenon, which has been discussed in another place.

[83] Dean. *Zeitschr. f. Imm.*, I, Vol. 13, 1912.
[84] Sachs. *Deut. med. Woch.*, 1905, No. 18.

To summarize, then, we have learned that there are a number of varieties of specific alexin absorption or fixation processes, one that is exerted by cells treated with specific sensitizer, be they blood or bacterial, the other that which occurs when unformed protein is brought into contact with its specific antiserum. The latter has been correlated with the precipitin reaction, in that it has been found that, whenever a specific precipitate is formed in such reactions, it is this precipitate on which the fixation depends. On the other hand, it is necessary to note that the formation of a precipitate is by no means necessary for the fixation, for, as is well known, if a series of precipitin tubes are set up, in each successive one of which the amount of antigen is diminished, a degree of dilution will soon be reached at which no visible precipitate will occur, but which nevertheless will show alexin fixation. The following is an illustration of such an experiment:

Sheep serum + antisheep serum	Precipitate	Fixation of 0.5 c. c. guinea pig complement
0.5 c. c. (1:20) + 0.5 c. c.	+	Complete
0.5 c. c. (1:50) 0.5 c. c.	++	Complete
0.5 c. c. (1:100) 0.5 c. c.	+++	Complete
0.5 c. c. (1:200) 0.5 c. c.	+++	Complete
0.5 c. c. (1:500) 0.5 c. c.	+++	Complete
0.5 c. c. (1:1,000) 0.5 c. c.	+	Complete
0.5 c. c. (1:2,000) 0.5 c. c.	+	Complete
0.5 c. c. (1:5,000) 0.5 c. c.	....	Partial
0.5 c. c. (1:10,000) 0.5 c. c.	....	Partial
0.5 c. c. (1:20,000) 0.5 c. c.	....	None

From such experiments it follows moreover that the fixation of alexin, carefully titrated, is a more delicate method of determining the presence of an antigen or, vice versa, of an antibody than is the observation of a visible precipitate, a fact which has been made use of, as we have mentioned, by Neisser and Sachs and others for forensic antigen determinations.

It should also be remembered that, if to such a precipitate there is added an excess of the antigen, the precipitate may be partially dissolved, and this dissolved precipitate, as Gay[85] has shown, may possess fixation properties. This, too, accounts for the fact, observed by a number of workers, that if, in a series of precipitin tests the supernatant fluids and the washed precipitates are separately examined for alexin fixation, the fixation properties reside entirely in the precipitates except in those tubes in which a considerable excess of antigen was used and in which, as in tubes 1 and 2 of the preceding

[85] Gay. *Univ. of Cal. Public. in Pathol.*, Vol. 2, No. 1, 1911.

protocol, the precipitates were relatively slight. The subject, though involved, is worthy of detailed consideration in this place since it seems to us to have an important bearing on certain theoretical conceptions which will be taken up below.

The important question now arises: what is the nature of the alexin fixation by the complexes formed by unformed proteins with their antibodies and, more especially, what is the nature of the alexin fixation exerted by specific precipitates? There have been much experimentation and speculation concerning this, and a number of different views are held. Gengou assumed, as we have seen, that this fixation, as studied by him, was entirely analogous to the fixation by sensitized bacterial or blood cells. He expressed the belief that treatment of an animal with an unformed protein produced not only specific precipitins but also specific sensitizers, analogous to those produced in response to treatment with bacterial or other cells. He noticed the parallelism between the quantity of the precipitate formed and the alexin fixation, but did not associate the two processes.

His conception of specific antiprotein sensitizers was accepted by a number of workers, and Wassermann and Bruck,[86] Friedberger[87] and several others brought out the facts that actual precipitate formation is not a necessary criterion of fixation. Thus the last-named writer showed that the precipitating power of a serum may be destroyed by moderate heat without a corresponding destruction of its fixing property. A similar independence of the precipitation from the complement-fixing property, in the presence of an antigen, has been observed by Muir and Martin.[88]

Gay,[89] also, though he was the first definitely to associate precipitin formation with the alexin-fixing property and, indeed, determined a rough parallelism between the amount of precipitate and the degree of alexin fixation, has nevertheless recently declared himself in favor of the assumption of the presence in protein antisera of two antibodies, the alexin-fixing lysins and the precipitins. This he does on the basis of certain experiments from which he concludes that the antigen-antibody complex which fixes alexin is distinct from the precipitin-precipitinogen complex, but is usually "brought down in its formation in such a way as to simulate fixation by the precipitate." Nicolle[90] goes even further than this in declaring that the "coagulins" or precipitins are "anticorps bons," which prevent the action of the albuminolysin upon the antigen, thereby inhibiting the liberation of poisonous cleavage products.

[86] Wassermann and Bruck. *Mediz. Kl.*, 1905, Vol. 1, No. 55.
[87] Friedberger. *Deut. med. Woch.*, 1906, No. 15.
[88] Muir and Martin. *Jour. Hyg.*, Vol. 6, 1906.
[89] Gay. *Loc. cit.* Also *Univ. of Cal. Publ. in Pathol.*, Vol. 2, No. 1, 1911.
[90] Nicolle. Ref. in *Bull. de l'Inst. Past.*, Vol. 5, 1907.

It seems to the writer [91] that the assumption of a separation between the precipitin and the albuminolysin is a needlessly complicated interpretation of the phenomena.

Without going into further complicated detail, it would seem to us [92] to be justified that we look upon the so-called precipitins not as separate antibodies but as identical with so-called albuminolysins. They unite with the antigen, producing an alexin-fixing complex. Since both reacting bodies are colloidal in nature, they precipitate each other in the test tube, but, following the laws governing other mutually precipitating colloids, they do so only when brought together in concentrations which lie within definite zones of relative proportions. The visible precipitation would seem, therefore, to be merely a secondary phenomenon, the essential one being the union of an antigen with a sensitizer by which it is rendered amenable to the action of the alexin. This would enable us to comprehend also the experiments of Friedberger, discussed in the section on anaphylaxis, in which it was shown that the action of alexin upon precipitates gives rise to the formation of toxic bodies just as this occurs when alexin acts upon sensitized cells.

Dean,[93] who has lately analyzed the relation between precipitation and alexin fixation on the basis of extensive experimentation, comes to the conclusion that the proportions of antigen and antibody which are favorable for rapid and complete precipitation do not favor the most complete alexin fixation. He states that the two reactions do not run a parallel course but believes that this does not mean that they are necessarily distinct phenomena. He says: "They represent two phases of the same reaction . . . a flocculent precipitate represents the final stage of a change which can be recognized in its earliest and incomplete stage by means of a complement fixation."

Our view differs from this only in that we believe that the precipitation is merely a secondary, colloidal phenomenon, which may, or may not, coincide with the phase of greatest alexin fixation, according to other fortuitous conditions which may favor or retard flocculation. Indeed, if our view be accepted, rapid compact precipitation may possibly be assumed to interfere with alexin fixation in that it would inhibit perfect contact of the alexin with the antigen-antibody complexes.

Dean finds that the alexin fixation in mixtures of antigen and antibody is quantitatively determined very largely by the character of the precipitation. If antigen and antibody are mixed in such proportions that a heavy precipitate is rapidly formed, there is either a very slight or no fixation of alexin, and there is, consequently, no

[91] Zinsser. *Jour. Exp. Med.*, Vol. 15, 1912. Also *Proc. of Soc. of Exp. Biol. and Med.*, April, 1913.
[92] Zinsser. *Jour. Exp. Med.*, Sept., 1913.
[93] Dean. *Zeitschr. f. Imm.*, Vol. 13, 1912.

relationship whatever between the amount of the precipitate and the amount of alexin fixed. If, on the other hand, antigen and antibody are mixed in such proportions that the turbidity develops slowly, as a very fine and gradual flocculation, large amounts of alexin may be fixed. The relationship is such a delicate one that if, in a series of experiments, varying amounts of antigen are mixed with a constant amount of antibody, two definite points may be found, at one of which there is the heaviest precipitation, and at the other the most intense alexin fixation. The amount at which the alexin fixation is most effective is usually the one at which there is materially less antigen than at the other. The necessity for optimum relative amounts for both reactions has been briefly touched upon in a discussion of the Neisser-Wechsberg phenomenon a few pages above. In cases in which an excess of antigen produces incomplete precipitation, no alexin fixation can be expected, according to Dean. In experiments in which antiserum and antigen are mixed in optimum relative amounts, the degree of alexin fixation is proportionate to the amount of antiserum used. Dean believes that alexin fixation probably occurs during the earliest stages of the precipitation, when the particles of precipitate are extremely small (perhaps because of the high surface tension), and that after the stage at which a definite turbidity is observed, very little alexin fixation takes place. Therefore, in order to obtain the maximum amount of alexin fixation, the alexin should be present in the tubes at the time when antigen meets antibody. Subsequent addition usually results in less fixation.

These considerations of Dean which have been confirmed generally in many of their aspects, are of the utmost importance to all workers with complement fixation, since neglecting them may reverse the results of experimental procedures.

Another view of the mechanism of alexin fixation is that which has been advanced by Neufeld and Haendel.[94]

These workers have found that sensitized cholera spirilla will fix hemolytic complement at 0° C., whereas the same bacteria at 37° C. will fix both the hemolytic and the bactericidal complement. They conclude from this that the fixation at 37° C. was brought about by virtue of the bactericidal amboceptor, whereas at 0° C. fixation was brought about by an antibody which is distinct from amboceptor or sensitizer. They believe from this and other observations, which we cannot consider in detail, that alexin fixation may be brought about by a special fixing antibody, the "Bordetscher Antikörper," which is not identical with any of the other known antibodies.[95]

Non-specific Alexin Fixation.—In all experiments which deal with alexin fixation by specific antigen-antibody complexes it is of the

[94] Neufeld and Haendel. *Arb. a. d. kais. Gesund.*, Vol. 28, 1908.

[95] As will be seen in a later section we are in entire disagreement with this view and believe that Dean's conception is the correct one.

greatest importance that we should guard against the errors easily introduced by fortuitous non-specific antihemolytic agencies. Thus there are a number of factors which will interfere with the functionation of alexin upon a sensitized antigen, either by direct non-specific absorption of the alexin itself or by producing physical conditions in the presence of which alexin cannot act.

Thus many animal tissue cells, in emulsion, will absorb alexin, and the same property may be possessed by tissue extracts. Von Dungern [96] was the first to call attention to this, and his observations have been variously confirmed. Muir [97] showed that the stromata of hemolyzed red blood cells exert strong anticomplementary action, and that this is due to a firm union with the complement. It is not unlikely that the action of cells in this respect is referable to their lipoidal contents. This suggestion was first made by Landsteiner and von Eisler,[98] who found that the petroleum-ether extracts of red blood cells possessed strong anticomplementary action which, to a limited extent, was specific toward the particular corpuscles from which the etracts had been made. Similar observations have been made by Noguchi,[99] who speaks of the substance he extracts as "protectin." In general, the protective action of the lipoidal extracts seems to depend largely upon cholesterin, and, since this substance is present to some extent in many tissues, their antihemolytic action is easily understood. In another section we have discussed the similar neutralizing action of lipoidal substances upon poisons of various kinds (saponin, tetanolysin, and snake poison, but, as we have noted there, the neutralizing properties of the extracts do not, as a rule, equal those of the whole tissues.[100] It is not unlikely that in such cases as Landsteiner suggests the potent agent is not the lipoid itself but rather a lipoid-protein combination, a class of substances of which we know very little, but the importance of which, in many phases of serum reactions, seems assured.

We have already mentioned that yeast cells may absorb alexin. And it has been found by Wilde [101] and others that almost all bacteria in emulsion may possess varying degrees of alexin-fixing properties even though unsensitized. There seems to be no regularity either qualitatively or quantitatively in regard to this, but the fixation is usually sufficiently marked to render the use of whole bacteria unreliable for specific fixation experiments. For this reason, as we will see, bacterial extracts must be used in such work unless careful quantitative controls are made. Upon what this fixation depends it is difficult to determine. It may be that it is purely non-specific and

[96] Von Dungern. *Münch. med. Woch.*, Nos. 20 and 28, 1900.
[97] Muir. "Studies in Immunity," London, Vol. 19.
[98] Landsteiner and von Eisler. *Wien. klin. Woch.*, No. 24, 1904.
[99] Noguchi. *Jour. Exp. Med.*, Vol. 8, 1906, p. 726.
[100] See also Ivar Bang, "Biochemie der Lipoide."
[101] Wilde. *Berl. klin. Woch.*, 1901, Vol. 38, and *Archiv f. Hyg.*, 39, 1902.

due to absorption of the fine emulsion of the bacteria comparable to that observed on the part of kaolin or quartz sand emulsions, or, possibly fixation by such bacterial emulsions may occur because of the small amounts of normal sensitizer almost always present in the serum employed as alexin.

Apart from the lipoids, a number of other substances have been found to fix alexin and exert consequent antihemolytic action. Thus Landsteiner and Stankovic,[102] and Landsteiner and von Eisler [103] describe the anti-alexic action of various proteins coagulated or precipitated. They refer this action not to particular chemical structure but to the colloidal state, since they obtained similar antilytic action with such inorganic emulsions as quartz sand and kaolin (aluminium-orthosilicate). Since anticomplementary action has, moreover, been noted in the case of a large number of extracts of such materials as wool, leather, etc., it is clear that the methods of alexin fixation, as applied to the forensic differentiation of blood, must be carefully controlled with this point in view.[104]

Among the most practically important non-specific agencies which fix alexin there are some which appear under certain conditions in normal serum. Noguchi [105] has found that serum will often develop anticomplementary properties as a consequence of heating during the process of inactivation. On more detailed investigation he determined that the anticomplementary action increased as the serum was heated to about 90° C. Above this temperature it is destroyed. He refers this property to the serum lipoids, since he was able to remove it by extraction with ether, the ether extract possessing the same anticomplementary power as the original serum before extraction.

Neisser and Döring [106] have noticed anti-alexic or anticomplementary properties of human sera which were destroyed on heating, and which they associate with disease of the kidneys, since they noted it in sera of uremic patients. Browning and McKenzie [107] have observed a similar heat-sensitive anti-alexic action on the part of normal serum, and the subject has been studied by Zinsser and Johnston.[108] It was found that all normal sera will develop anti-alexic properties on preservation at room temperature within a few days, and more slowly but no less regularly in the ice chest. This anti-alexin is destroyed on heating to 56° C., and may be precipitated out with the globulins of the serum. There appeared in these studies

[102] Landsteiner and Stankovic. *Centralbl. f. Bakt.*, 1906, Vols. 41 and 42.

[103] Landsteiner and von Eisler. *Wien. klin. Woch.*, 1904, No. 24.

[104] Uhlenhuth. *Deut. med. Woch.*, 1906, Nos. 31 and 51, and *Centralbl. f. Bakt.*, 1906, I, Ref., Vol. 38.

[105] Noguchi. *Jour. Exp. Med.*, Vol. 8, 1906, p. 726.

[106] Neisser and Döring. *Berl. klin. Woch.*, 1901, No. 22.

[107] Browning and McKenzie. *Jour. Path. and Bact.*, Vol. 13, 1909.

[108] Zinsser and Johnston. *Jour. Exp. Med.*, Vol. 13, 1911.

no particular association between the anti-alexic property and nephritis.

The action of alexin upon sensitized cells may be prevented, also, by physical or chemical conditions without actual fixation or binding of the alexin. We refer to the effects of the addition of salts, problems which have been considered above.

CHAPTER VIII

PRACTICAL APPLICATIONS OF THE COMPLEMENT-FIXATION METHOD

THE WASSERMANN REACTION

The principle of specific alexin fixation has been practically utilized in the diagnosis of disease and in the forensic determination of the nature of spots of blood or other protein material.

Soon after Bordet and Gengou's experiments Wassermann and Bruck [1] showed that bacterial extracts could be successfully substituted for whole bacteria in these reactions. Citron,[2] too, made similar observations, and, indeed, we now know that the use of bacterial extracts is more suitable for these experiments than are emulsions of whole bacteria, since, as we have mentioned above, bacterial emulsions may often fix small amounts of complement of themselves (without specific sensitization), thereby confusing the results of the reaction.

On the basis of their experience with bacterial extracts Wassermann and Bruck [3] then determined that complement fixation could be carried out in tuberculosis when the various tuberculin preparations were used as antigen.[4] These investigations fell into the period during which active research upon the *Spirochœta pallida* in syphilis was going on, and it occurred to Wassermann that the technique of complement or alexin fixation might be utilized in the diagnosis of syphilis. Together with Neisser and Bruck [5] he subjected this idea to experimental test. The publication of their first results appeared in 1906. They used in their experiments the syphilitic monkeys which were being observed in Neisser's clinic. Their method consisted in mixing inactivated serum from syphilis-inoculated monkeys with organ extracts, serum, etc., of syphilitic human beings, and adding a small amount of fresh guinea pig complement. After these materials had been together for a certain time, sensitized red blood

[1] Wassermann and Bruck. *Med. Klinik,* Vol. 55, 1905.

[2] Citron. *Centralbl. f. Bakt.,* Vol. 41, 1906.

[3] Wassermann and Bruck. *Deut. med. Woch.,* No. 12, 1906.

[4] Complement fixation in tuberculosis is not yet on a practical or reliable basis. Recent claims of Besredka (*Ann. Past.,* 1913) for his new antigen promise a successful technique, but no extensive confirmation has followed up to the present time.

[5] Wassermann, A. Neisser, and Bruck. *Deut. med. Woch.,* No. 19, 1906.

cells were added. If the complement was bound during the first exposure no hemolysis resulted and the reaction was regarded as positive. From their results they drew the following conclusions:

1. Immune serum from monkeys, produced by treatment with syphilitic material, will sensitize syphilitic material from human beings or monkeys, so that an alexin-fixing complex is formed.

2. Complement fixation results only when the syphilitic immune serum of monkeys is added to similar material from men or monkeys, but not when added to organ extracts of normal men or monkeys.

3. Normal monkey serum has no such action.

They concluded that their results justified them in assuming a specific fixation due to specific antisyphilitic immune bodies in the blood of the treated monkeys. They excluded experimentally the possibility of fixation by a precipitin reaction resulting from the treatment of the monkeys with human material. It might well have happened that precipitins against human protein appearing in the serum of the treated monkeys might subsequently react with the human protein material used as antigen, a complement-fixing complex resulting. This, however, was excluded by the fact that they obtained positive reactions only when the human material was obtained from luetic lesions.

The same authors, with Schucht,[6] very soon after this, extended their method to the diagnosis of syphilis in human beings. The same thing had been done shortly before their publication appeared by Detre [7] on a smaller material. By these and many other investigations it was very soon shown that syphilis may be reliably diagnosed by complement fixation when extracts of the syphilitic organs, employed as antigen, are mixed with the inactivated serum of syphilitic individuals. It was incidentally shown by Wassermann and Plaut [8] that the reaction could be obtained not only with blood serum but also with spinal fluid in paralytic cases.

It was generally assumed, at this time, that the reaction in syphilis depended, as in the case of other infections, upon the presence in the syphilitic serum of specific antibodies. For it seemed reasonable to suppose that the specific antigen obtained in the extracts was derived from the extraction of large numbers of spirochetes demonstrable in the extracted organs.

This, of course, is the most logical and simple theoretical conception of the reaction, and is justified on the basis of analogy. Unfortunately, however, it was soon found by a number of workers,

[6] Wassermann, Neisser, Bruck, and Schucht. *Zeitschr. f. Hyg.*, Vol. 55, 1906.

[7] Detre. *Wien. kl. Woch.*, Vol. 19, No. 21, 1906.

[8] Wassermann and Plaut. *Deut. med. Woch.*, No. 44, 1906.

Marie and Levaditi,[9] Weygant, Kraus and Volk, Landsteiner, Müller, and Pötzl,[10] and others that antigens perfectly capable of fixing complement in the presence of syphilitic serum could be produced from normal organs.[11]

Theoretically it must be admitted that we are very much in the dark at present. The fact, now entirely unquestionable, that the sera of syphilitic patients will give fixation with antigens derived from extracts of normal organs, as well as from those of syphilitic organs, seems to throw doubt upon the simple specific antigen-antibody conception at first held.

In order to understand the questions involved in the theories of the Wassermann reaction as at present conceived it will be necessary to consider the types of antigen which are now employed.

Wassermann's original method of antigen preparation consisted in using the liver or spleen of a congenitally syphilitic fetus. The organs were finely divided and emulsified in 4 to 6 parts of normal salt solution. This mixture was shaken for 24 hours, centrifugalized, and the clear supernatant fluid used as antigen. Later the specific organ substances were extracted by Porges and Meier [12] in five times the volume of absolute alcohol for 24 hours. This alcoholic extract was evaporated *in vacuo* and the residue taken up in salt solution and shaken until an even suspension resulted.

After it had been discovered that normal organ extracts could serve as antigen as well as the extracts of syphilitic organs, Landsteiner, Porges and Meier, and others, introduced antigens produced by alcoholic extraction of normal organs of animals and of man. Landsteiner introduced the alcoholic extract of normal guinea pig organs, especially extracts of the heart and liver, and Weil and Braun [13] made use of extracts of normal human organs. There are various methods of preparing extracts for this purpose. We may mention, to illustrate these methods, the one suggested, first, we believe, by Noguchi, a procedure which is applicable to the extraction of normal human organs (spleen), beef hearts, and guinea pig hearts. The finely divided or triturated organ substance is shaken up with five times its weight of absolute alcohol and allowed to stand in the incubator at 37.5° C., for from 5 to 7 days. At the end of this time it is filtered through cheesecloth and then through coarse paper, and

[9] Marie and Levaditi. Cited from McIntosh and Fildes' "Syphilis." Longmans & Co., 1911, p. 94.

[10] Landsteiner, Müller, and Pötzl. *Wien. kl. Woch.*, Vol. 20, 1907.

[11] An extensive historical review of the development of the Wassermann reaction is found in the book of Boas, "Die Wassermannsche Reaktion," Karger, Berlin, 1911. Since these earlier publications have appeared the literature of the Wassermann reaction has become very extensive. It is enumerated more fully than we can afford space for here in the book of Noguchi ("Serum Diagnosis of Syphilis") and that of Boas, mentioned above.

[12] Porges and Meier. *Berl. kl. Woch.*, No. 15, 1908.

[13] Weil and Braun. *Berl. kl. Woch.*, No. 49, 1907.

the filtrate placed in a large crystallizing dish in which it is evaporated at room temperature with the aid of an electric fan. A gummy yellow residue is left, which is then taken up in as small a quantity of ether as possible. This ether solution is then precipitated with 4 times its volume of acetone, in consequence of which there is a profuse precipitation of coarse white flakes. This acetone-insoluble substance, which is at first white, later yellowish, in color, is the stock antigen. A little of this is taken up in a very small quantity of ether, and this ethereal solution is shaken up in salt solution until the ether has evaporated or the material has gone into very fine colloidal suspension in the salt solution. This is the antigen ready to be used.

It is immediately evident that these antigenic substances must consist very largely of lipoidal extractives of the organ substances, and it has been found that such antigen contains sodium oleate, lecithin and cholesterin. Indeed, Porges and Meier have claimed that a 1 per cent. solution of commercial lecithin may be used with success. Browning and Cruikshank [14] have found further that the addition of small amounts of cholesterin to syphilitic antigen very largely increases its specifically diagnostic value, and this idea has since been utilized more especially by Sachs,[15] Walker and Swift,[16] and others. Sachs, especially, has obtained excellent antigens in the following way: 1 gram of moist guinea pig heart substance was extracted with 5 c. c. of alcohol and left at room temperature for twelve hours or in the ice box for two days; it was then filtered and 0.5 to 1 per cent. of cholesterin was added; frequently the alcohol extract had to be diluted two or three times before use. Sachs and Rondoni [17] have also recommended artificial mixtures of lipoids containing sodium oleate, lecithin, and oleic acid.

The fact that cholesterin added to alcoholic organ extracts increases the antigenic value of these for the Wassermann reaction is all the more curious inasmuch as cholesterin alone has practically no antigenic action. Walker and Swift have recommended an antigen in which alcoholic extracts of human or guinea pig hearts were made up to 0.4 per cent. of cholesterin, 0.4 per cent. having been found by comparative test to be the most favorable concentration. Cholesterin-liver extracts or even alcoholic extracts of syphilitic livers without cholesterin were found to be inferior in specific antigenic value to 0.4 per cent. cholesterin-heart antigens. From the experience of many investigators it now seems unquestionable that additions of cholesterin increase the delicacy of the reaction in that more cases react positively with such an antigen than with the uncholesterinized

[14] Browning and Cruikshank. *Jour. of Path. and Bact.*, Vol. 16, 1911.
[15] Sachs. *Berl. kl. Woch.*, No. 46, 1911.
[16] Walker and Swift. *Jour. of Exp. Med.*, Vol. 18, 1913.
[17] Sachs and Rondoni. *Zeitschr. f. Imm.*, Vol. 1, 1909.

preparations. The experience of Hopkins and Zimmermann, however, would indicate that great caution must be exercised when the reaction is done in this way, since occasional positive results are obtained with cases clinically not syphilitic. These workers believe that cholesterinized antigen is extremely useful, but advise its use only parallel with the ordinary lipoidal antigens and together with careful study of the clinical aspects of the case.

The fact that these antigens are non-specific in origin naturally necessitates careful determination of their usefulness before they are used. Before any antigen can be regarded as reliable, therefore, a titration must be carried out in the following way: Two series of tubes are prepared, in the first of which antigen and complement are added to normal serum, and in the second the same substances are added to known syphilitic serum. The antigen must, of course, be such that in no test tube does it cause alexin fixation in the presence of normal serum, but, in the quantities used, it must give fixation regularly with syphilitic serum. An example of such a titration may be tabulated as follows:

Example of Antigen Titration

Antigen by Landsteiner's method: normal guinea pig heart freed from fat and ground up in a mortar. To each gram is added 5 c. c. of absolute ethyl alcohol and the mixture allowed to extract at 60° C. for 12 hours (or several days at 37.5° C.). It is then filtered through paper. The following titration is then carried out:

A	Tube 1	Tube 2	Tube 3	Tube 4	Tube 5
Normal serum	0.2	0.2	0.2	0.2	—
Antigen	*0.05*	*0.1*	*0.2*	*0.3*	*0.6*
Alexin	0.1	0.1	0.1	0.1	0.1
B	**Tube 1**	**Tube 2**	**Tube 3**	**Tube 4**	
Syphilitic serum	0.2	0.2	0.2	0.2	
Antigen	*0.05*	*0.1*	*0.2*	*0.3*	
Alexin	0.1	0.1	0.1	0.1	

The volume in all of these tubes is brought to 3 c. c. with isotonic salt solution. After one hour at 37.5° C., sensitized red cells are added to each tube.[18] If the antigen is suitable in that it does not

[18] Tube "5" is the antigen control which shows that the antigen in large amounts is neither anticomplementary nor hemolytic by itself. It is well, in addition, also to test out various amounts of the antigen and alexin, without

fix alexin by itself or in the presence of normal serum, hemolysis will result in all of the tubes of series A. If it is suitable in that it fixes in the presence of syphilitic serum, the tubes in series B will show no hemolysis; if there is slight hemolysis in B 1, it is inferred that 0.05 c. c. of the antigen is insufficient, and the smallest amount (0.1 c. c.), which completely fixes 0.1 c. c. of alexin in the presence of the positive serum, is the quantity used. Again the antigen may be able to cause hemolysis by itself if used in too large amounts. If this is the case in tube B 4, then this antigen is suitable only in amounts varying between 0.1 c. c. and 0.2 c. c.

The titration is done with varying quantities because too little antigen might fail in fixing the alexin, even if the serum were positively syphilitic, whereas too much antigen might possess alexin-fixing properties in itself, even in the presence of normal serum, or possibly without any serum at all, an attribute which is not uncommonly possessed by lipoidal extracts.

It is thus seen that Wassermann reactions can be carried out with antigens which do not contain extracts of syphilitic lesions or of the micro-organisms which give rise to syphilis. This fact alone would exclude the possibility of considering the fixation of complement as at present carried out in the Wassermann reaction as being due to a specific antigen-antibody union.

This conclusion is strengthened by the recent discovery that a specific antigen prepared from cultures of *Spirochæta pallida* cannot be successfully used in diagnostic Wassermann tests. The first investigations of this kind were made by Schereschewsky,[19] who used as antigen extracts of mixed cultures in which the spirochete was present; his results were inconclusive. Noguchi [20] later investigated this phase of the problem, preparing his antigens by the extraction of pure cultures and of syphilitic rabbit testicles in which the spirochetes were very profuse. He found that positive tests with such an antigen were obtained only in isolated cases of prolonged syphilis which had been thoroughly treated, and that the ordinary Wassermann reaction, as obtained in active cases, is not due to antibodies which combine specifically with the pallida antigen. Craig and Nichols [21] also have found that cases of untreated syphilis which gave positive reactions with syphilitic liver extracts gave absolutely negative results when culture antigens were used.

From these results also we may infer that the Wassermann reaction does not represent a fixation of alexin by the union of a specific syphilitic antigen either normal or syphilitic serum, to determine the largest amount of antigen which, by itself, is devoid of the actions mentioned above.

[19] Schereschewsky. *Deut. med. Woch.*, 1909, p. 1653.
[20] Noguchi. *Jour. A. M. A.*, Vol. 58, 1912.
[21] Craig and Nichols. *Jour. of Exp. Med.*, Vol. 16, 1912.

with antibodies found against the *Spirochæta pallida*. Noguchi concludes that it is caused by "lipotropic" substances in the sera of syphilitic human beings; a conclusion which is justified by the fact that the antigens used, all of them, contain large quantities of lipoids. It must be acknowledged, however, that we have no definite information concerning the nature of the reaction beyond this. Schmidt [22] believes that it is a colloidal reaction, and depends upon the union of the serum globulins with the extract colloids in the antigen. In normal serum such a union is prevented by the albumins which act as a sort of protective colloid. In syphilitic serum the globulins are increased quantitatively or are changed qualitatively in the degree of their dispersion, or possibly in both characteristics. He regards the serum globulins in the Wassermann reaction as directly uniting with the extract colloid.

Levaditi and Yamanouchi [23] also conclude that the Wassermann reaction depends upon the union of two colloidal substances—one a non-proteid constituent of syphilitic serum (cholesterin derivatives or fatty acids), the other the lipoidal constituents of the antigen. Like others they found that the active substances in the antigenic extracts are non-protein and alcohol soluble.

It is interesting to note, moreover, that Porges and Meier [24] observed actual precipitation when syphilitic serum was added to lecithin emulsions. In consequence, attempts have been made to make the diagnosis of syphilis by direct precipitation of syphilitic serum by such emulsions of lecithin and of sodium glycocholate (Merck). The results of these investigations as well as those of Klausner,[25] who claims that syphilitic sera are more easily precipitated by distilled water than are normal sera, have led to no diagnostically reliable results, but they have seemed to show that the serum globulins are probably more plentiful and more easily precipitated out of syphilitic than out of normal sera.

The inference of many workers, therefore, has been that the Wassermann reaction is primarily due to the precipitation of (probably) globulin by the lipoidal colloids of the antigen, the resulting precipitate being capable of absorbing alexin. Jacobsthal [26] has examined mixtures of syphilitic serum and antigen by the ultramicroscopic method, and claims that precipitates are always present even when they are not macroscopically visible. Bergel,[27] who has recently suggested the importance of specific lipase production as a cause of hemolysis, suggests that the Wassermann reaction is due to fixation exerted by the products of the action of a specific lipase formed in the syphilitic body against "lues-lipoids." This theory is open to objections similar to those mentioned above, namely, that the antigen need not necessarily be a lues-lipoid, but may be derived from normal organs. Other theories have been brought forward by Bruck, Weil, Braun, Manwaring, and more recently by Rabinowitch.[28] The data supporting most of these theories are, as yet, too speculative to justify our discussion of them at any length. The only fact which seems established with any reasonable certainty is the independence of the Wassermann test from a specific antigen-antibody reaction in the usual sense.

Another suggestion which has been made, particularly by Weil and

[22] Schmidt. *Zeitschr. f. Hyg.*, Vol. 69, 1911.

[23] Levaditi and Yamanouchi. *C. R. de la Soc. de Biol.*, 1907, Vol. 63, p. 740.

[24] Porges and Meier. *Berl. kl. Woch.*, No. 15, 1908.

[25] Klausner. *Wien. kl. Woch.*, No. 7, 1908.

[26] Jacobsthal. *Münch. med. Woch.*, 1910.

[27] Bergel. *Zeitschr. f. Imm.*, Vol. 17, 1913.

[28] Rabinowitch. *Centralbl. f. Bakt.*, Orig., 1914.

Brown,[29] is that the antibody involved in syphilis is an auto-antibody, resulting probably from the destruction of tissue by the syphilitic infection, with the liberation of lipoid-protein complexes against which the body, itself, then reacts with antibody formation. Such lipoid-protein substances, they suppose, are present in small amounts in normal tissues, but are enormously increased in the course of syphilitic infection. There is not much experimental or theoretical basis for the opinion of Weil and Brown.

While, therefore, we are still considerably in the dark concerning the true nature of the Wassermann reaction, we may state with safety that there is present in the syphilitic serum a substance which leads to the formation of a precipitate when brought into contact with properly prepared alcoholic extracts of normal tissues. The so-called antigenic substances, then, are probably lipoidal in nature, or, at any rate, represent lipoid-protein complexes. The precipitate formed, probably because of its physical properties, is now capable of fixing alexin.

Although the Wassermann reaction is thus apparently not based on those principles in the investigation of which it was discovered, its practical diagnostic value is not therefore diminished. For its proper performance any of the methods of antigen preparation considered above may be employed, provided that the usefulness of the preparation utilized is carefully controlled in each case as indicated. Since, of course, a hemolytic system is used in such tests as an indicator, it is necessary also to titrate sensitizer and alexin.

From what has been said in another place concerning the quantitative relations of alexin and amboceptor or sensitizer (see reference to work of Morgenroth and Sachs, p. 163), it is evident that the use of too strongly sensitized cells might result in hemolysis, if a slight fraction of alexin were left unbound by a weak syphilis reaction. Conversely the use of too large a quantity of alexin would result in hemolysis, since, even if the amount of syphilitic fixation were considerable, a sufficient excess of alexin might remain. The use of uniform amounts of fresh guinea pig serum in each case does not control this adequately, for different specimens of guinea pig serum may vary considerably in alexin content. In consequence, titrations of both sensitizer and alexin should be made. For practical purposes it is quite enough to titrate the hemolytic sensitizer every few weeks and use a stated amount in successive reactions. The alexin or complement can then be titrated individually for each set of reactions. Examples of such preliminary titrations follow:

Titration of Hemolytic Amboceptor or Sensitizer

Rabbit injected 3 times at 5-day intervals with washed sheep corpuscles . . ., 3, 4, and 5 c. c., and bled 10 days after the last injection.[30]

[29] Weil and Brown. *Berl. klin. Woch.*, 44, 1907, 1570. Cited from Bruck, "Serodiagnose der Syphilis," p. 618.

[30] In immunizing animals with blood cells for this or any other purpose

This serum is inactivated at 56° C. for 20 minutes.

	Washed sheep corpuscles 5% emulsion in salt solution	Sensitizer	Fresh g. p. serum	Hemolysis
1	1 c. c.	0.01	0.1	+++
2	1 c. c.	0.005	0.1	+++
3	1 c. c.	0.003	0.1	+++
4	1 c. c.	0.001	0.1	+++
5	1 c. c.	0.0005	0.1	++
6	1 c. c.	0.0002	0.1	±
7	1 c. c.	....	0.1	....
8	1 c. c.	salt sol.	....	....

In this case 0.001 c. c. still causes complete hemolysis of 1 c. c. of a 5 per cent. emulsion of sheep cells (volumetric measurement of cells sedimented in the centrifuge), and this amount ($\frac{1}{1000}$ c. c.) is called the "hemolytic unit" of sensitizer; two units are then used in the reactions.

Against these cells alexin can, in each case, be titrated as follows:

Alexin Titration:

Fresh Guinea Pig Serum Pipetted from Clot

	Red cells 5% emulsion	Sensitizer as above determined	Guinea pig serum	Hemolysis
1	1 c. c.	2 units (.002)	0.1 c. c.	+++
2	1 c. c.	2 units (.002)	0.05 c. c.	+++
3	1 c. c.	2 units (.002)	0.025 c. c.	±
4	1 c. c.	2 units (.002)	0.01 c. c.	......

The smallest amount of alexin which completely hemolyzes the red cells (0.05 in this case) is the amount used. Since it is easier to measure larger volumes with accuracy, the alexin is diluted 1 to 10 in salt solution before use. A typical Wassermann reaction can then be carried out as follows:

it is necessary to wash the cells very carefully in salt solution. Unless this is done blood serum or plasma will be injected with them and the treated animal will respond by the formation not only of hemolysin but of precipitins for the serum proteins as well. When a subsequent hemolytic test is carried out, a precipitin reaction between the precipitin in the antiserum and serum adhering to the corpuscles will follow, and this, as we have seen, will fix alexin, obscuring other reactions which may be under observation.

Scheme for Wassermann Test

ADAPTED TO ORIGINAL WASSERMANN SYSTEM AFTER SCHEME OF NOGUCHI

	Test with unknown serum	Test with known positive syphilitic serum	Test with known negative normal serum	Test without serum to control efficiency of hemolytic system
Back row without antigen	Serum .2 c. c. + O Complement .1 c. c. + Salt sol. 3 c. c. 2.	Total volume, 3 c. c. Serum .2 c. c. + O Complement .1 c. c. + Salt sol. 3 c. c. 4.	Serum .2 c. c. + O Complement .1 c. c. + Salt sol. 3 c. c. 6.	O Complement .1 c. c. + Salt sol. 3 c. c. 8.
Front row with antigen	Serum .2 c. c. + O Complement .1 c. c. + Antigen (required amount in 1 c. c. salt sol.) + Salt sol. 2 c. c. 1.	Total volume, 3 c. c. Serum .2 c. c. + O Complement .1 c. c. + Antigen + Salt sol. 2 c. c. 3.	Serum .2 c. c. + O Complement .1 c. c. + Antigen + Salt sol. 2 c. c. 5.	O Complement .1 c. c. + Antigen + Salt sol. 2 c. c. 7.

O = Test tube.

Place in water bath at 40° C. for one hour, then add to all tubes red blood cells and amboceptor. These are previously mixed so that 2 c. c. contains the equivalents of 1 c. c. of a 5 per cent. emulsion of sheep corpuscles and 2 units of amboceptor. Again expose to 40° C. If the serum tested is positive, tubes 1 and 3 should show no hemolysis, all the other tubes showing complete hemolysis in one hour.

Since many human sera normally contain small amounts of antisheep sensitizer, it is the habit of many workers to add the sheep corpuscles, without the sensitizer or amboceptor, and incubate for a half-hour. If, at the end of this time, no hemolysis has occurred either in the front or the back row, then amboceptor may be added. This technique avoids the possible error introduced by an excess of amboceptor, a condition which easily occurs when any large amount is normally present in the serum and in addition to this 2 units are added as in the test described above.

The above represents the typical "Wassermann" as at present carried out in most laboratories. It may be carried out just as well and with greater economy of material by using one-half the amounts throughout. It is evident that the performance of the reaction calls for experience of serum technique, and knowledge of such reactions, so that fortuitous irregularities may be intelligently controlled. It is our opinion that the performance of routine Wassermann tests by workers without a thorough knowledge of the fundamental facts of

serum phenomena is worse than useless in that insufficient attention to special conditions and to details may easily result in a positive reaction when syphilis is not present, and vice versa.

Recently Archibald McNeil and others have exposed the mixtures of complement, antigen, and patient's serum at refrigerator temperature for a number of hours instead of in the water bath or thermostat at 37.5° C., before adding the sensitized cells. It is a curious fact, which has not yet been satisfactorily explained, that such a procedure increases the delicacy of the reaction. It may be that, when the tubes containing the antigen, patient's serum, and alexin are left at incubator temperature, partial alexin fixation only can take place during the brief period of 30 minutes to one hour, which is usually employed. More prolonged exposure at this temperature would not be advisable on account of deterioration of the alexin. On the other hand, at ordinary ice-box temperatures of about 8° to 10° C., the exposure can be continued for as long as 10 hours without extensive complement deterioration, and meanwhile more complete fixation can occur. This so-called ice-box method has become a regular routine in many Wassermann laboratories. In our own department, it has been employed for a good many years in parallel with the water-bath tests, each serum being used for a water-bath reaction in which the cholesterinated antigen is used, as well as in an ice-box test with an ordinary alcoholic antigen without cholesterin. Whether or not it will be advisable to continue such duplication after further development of the direct precipitation methods of Sachs and Georgi, and of Kahn, remains to be seen.

Many modifications of the Wassermann test have been suggested. Probably the most important is that of Noguchi. The chief justification for this modification is the fact that many normal human sera contain hemolysins for sheep corpuscles. For this reason many workers carry out the ordinary Wassermann technique without adding antisheep sensitizer or amboceptor until they have first observed whether or not the tested serum (in the "back row," without antigen) will not hemolyze the corpuscles without such an addition, adding the sensitizer only when this does not take place. This is advisable since the presence of any considerable amount of normal antisheep sensitizer in the human serum which is being examined (if added to the amount used in the ordinary reaction, 2 units) may so increase the total quantity that hemolysis will result even after most of the alexin has been fixed. Noguchi excludes this uncertainty by avoiding the use of the "sheep cell-antisheep sensitizer" system entirely, substituting a hemolytic complex consisting of human cells and antihuman sensitizer, produced by injecting washed human corpuscles into rabbits.

His technique may be best illustrated in the following tabulation:

Reagents

1. Sensitizer prepared by injecting washed human blood corpuscles into rabbits.
2. 1 per cent. emulsion of washed human blood cells.
3. Alexin—fresh guinea pig serum diluted with one and one-half volumes of salt solution, 40 per cent.

The reaction is performed in the following way:

Noguchi's Method of Complement Fixation for the Serum Diagnosis of Syphilis

	Set for diagnosis Test with the serum in question	Positive control set Test with a positive syphilitic serum	Negative control set Test with a normal serum			
Rear row	a. Unknown serum, 1 drop* b. Complement, 2 units ○ c. Corpuscle susp., 1 c. c.	a. ′Positive syph. serum, 1 drop* b. Complement, 2 units ○ c. Corpuscle susp., 1 c. c.	a. ″Normal serum, 1 drop* b. Complement, 2 units ○ c. Corpuscle susp., 1 c. c.	Incubation at 37° C. for 1 hour.	Addition of antihuman amboceptor, 2 units to all tubes.	Incubation at 37° C. for 2 hours longer, then at room temperature.
Front row	a. Unknown serum, 1 drop* b. Complement, 2 units ○ c. Corpuscle susp., 1 c. c. +Antigen	a. ′Positive syph. serum, 1 drop* b. Complement, 2 units ○ c. Corpuscle susp., 1 c. c. +Antigen	a. ″Normal serum, 1 drop* b. Complement, 2 units ○ c. Corpuscle susp., 1 c. c. +Antigen			

* When working with inactivated serum 4 drops (0.08 c. c.) should be employed. With cerebrospinal fluid, 0.2 c. c. (not inactivated) is used.

(Taken from Noguchi's "Serum Diagnosis of Syphilis," Lippincott, 1910, p. 57.)

Bauer [31] has introduced a modification in which he utilizes the presence of normal sheep sensitizer in many human sera. He performs his tests without the addition of antisheep sensitizer at first, adding this only to those tubes in which controls have shown that no normal sensitizer is present. Stern,[32] on the other hand, utilizes the alexin normally present in human serum. The syphilitic serum to be tested is, therefore, not inactivated, and the sheep cells are more heavily sensitized (9 to 12 units). It seems to us that this method is objectionable chiefly because of the anticomplementary action which develops in most normal human sera if kept for a short time, and which can be removed only by inactivation.

Other modifications of the Wassermann reaction are those of

[31] Bauer. *Semaine Medicale,* 28, 1908.
[32] Stern. *Zeitschr. f. Imm.,* Vol. 1, 1909.

Jacobaeus [33] and of Wechselman.[34] It seems, however, that, as the reaction is gaining in importance in clinical diagnosis, most laboratories are adhering to the original system used by Wassermann and his associates, except for the substitution of the non-specific lipoidal antigens for the originally employed organ extracts.

The value of the Wassermann test in the diagnosis of the various stages of syphilis is a problem which can be approached only by careful statistical analysis of the results obtained. This has been done by various investigators, and some of the results have been tabulated in the books of Noguchi, of Boas, and of McIntosh and Fildes. The figures we cite are those largely taken from Boas, as summarized in F. C. Wood's "Chemical and Microscopical Diagnosis" (D. Appleton & Co., 1911), pp. 706 et seq.

Primary syphilis, 974 cases, 56.5 per cent. positive.

The reaction may appear before the primary sore, but this is very rare. Usually it is positive in from 5 to 6 weeks after infection.

Secondary syphilis, 2,762 cases, 88 per cent. positive. In untreated cases they are stated to be 100 per cent. positive.

Tertiary syphilis, 830 cases, 80 per cent. positive.

Tabes, 360 cases, 70 per cent. positive.

Dementia paralytica, 95 to 100 per cent. positive.

The tabulation on the following page, taken directly from Boas, will give a comprehensive summary of this phase of the problem.

Since the reaction is not a specific antigen-antibody union but depends on some substance liberated or produced by reason of the syphilitic injection, it is not out of question that other infections may give rise to a "positive Wassermann." And this, indeed, is the case. It was claimed for a time that a positive reaction may be obtained in tuberculosis, but this has been refuted by subsequent experience, and the earlier positive results probably depended upon faulty technique. There can be little doubt, however, that occasional positive reactions are obtained in cases of leprosy, scarlet fever, malaria, and trypanosoma infections.

The spinal fluid may be used instead of the blood serum in cases of syphilis of the central nervous system, but even here, as Citron [35] has shown, the results with blood serum are more frequently positive than those done with the spinal fluid itself. In isolated cases positive reactions have been obtained with ascitic fluids, pleural and pericardial exudates. Bab [36] reports a case of positive reaction in the milk of a syphilitic mother. Serum obtained at autopsy is not

[33] Jacobaeus. *Zeitschr. f. Imm.,* Vol. 8, 1911.
[34] Wechselman. *Zeitschr. f. Imm.,* Vol. 3, 1909.
[35] Citron. *Deut. med. Woch.,* 1907, No. 29, p. 1165.
[36] Bab. *Münch. med. Woch.,* Vol. 46, 1907.

Table Compiled by Boas, loc. cit., p. 138.

Stage of disease	Number of cases	Positive reaction	Negative reaction
Control cases (not syphilitic)	1,064	1 (scarlatina)	1,063
Induration	76	56	20
Secondary			
Early untreated	269	269	0
Recurrent after treatment	199	187	12
Tertiary			
No treatment of early tertiary manifestations	63	63	0
Treatment	20	16	4
Latent syphilis			
Within 3 yrs. after infection	243	89	154
After 3 yrs	111	44	87
Tabes			
Untreated	17	17	0
Treated	26	11	15
Dementia paralytica			
Serum	139	139	0
Spinal fluid	67	61	6
Congenital			
With symptoms	54	54	0
Without symptoms	10	7	3

suitable for the reaction, since this, for unknown reasons, may often give a positive reaction in non-syphilitic cases.

Direct Precipitation Methods in the Diagnosis of Syphilis.—In view of what we have said about the fixation of alexin by specific precipitates, it is interesting to know that the probable physical basis of the reaction consists in the actual formation of a precipitate by the union of the so-called antigenic substance and the reacting substance in the syphilitic serum. We have already mentioned that Jacobsthal in 1910 showed that a microscopic coagulation of particles could be detected in mixtures of syphilitic serum and lipoidal antigens under the ultra-microscope. This observation was confirmed by a considerable number of workers, and has borne fruit in a number of diagnostic tests in which syphilitic serum and antigen extracts produce macroscopically visible precipitates. The best known of these is that spoken of as the Sachs-Georgi reaction. The following description is one prepared for us by our associate, Dr. Frederic Parker, Jr., for our "Textbook of Bacteriology," [37] on the basis of comparative investigations made with this reaction and the Wassermann reaction upon a series of positive and negative cases.

[37] Hiss and Zinsser. "Textbook of Bacteriology," Fifth Edition, D. Appleton & Co., N. Y., 1922.

Sachs-Georgi Reaction for Syphilis (Direct Precipitation). Preparation of Extract.—A beef heart is freed from fat and endocardium, cut up finely and ground in a mortar. It is then shaken with 5 volumes of 95 per cent. alcohol and a few glass beads in a shaking machine for 5 hours, allowed to stand at room temperature over night, filtered through ordinary filter paper next morning, then placed in the ice-box for at least two days, when it is again filtered through ordinary filter paper, and is now ready for use. It must first be titrated against a standard extract on a number of sera to determine the optimum dilution and cholesterinization. For this, the alcoholic extract is diluted with 1, 2, and 3 parts of alcohol, and to fractions of each of these dilutions, 0.3, 0.45, 0.6 and 0.75 per cent. of a 1 per cent. alcoholic solution of cholesterin is added. These various portions are then diluted with 5 parts of saline as described below, and set up against a standard extract. At least two such extracts should be used in each test.

Extract Dilution.—The alcoholic cholesterinized extract is diluted with 5 parts of saline as follows: The required amount of extract is placed in an Erlenmeyer flask; to it is rapidly added from a burette an equal volume of saline. It is shaken gently and allowed to stand 10 minutes, then the remaining 4 volumes of saline are rapidly run in. It is again shaken and is ready for use.

Serum.—Serum should be as fresh as possible. Three or four days is not too old. A slight degree of hemolysis does not interfere. Before use in the test, it is heated for ½ hour at 55° to 56° C. and should not be used sooner than 3 hours after heating. Sachs and Georgi [38] recommend that spinal fluids should be used undiluted in amounts of 1 c. c. and 0.5 c. c.

Saline.—0.85 per cent. sodium chlorid in distilled water. Should be sterile and as fresh as possible.

Test.—0.1 c. c. serum is diluted with 0.9 c. c. saline and to this is added 0.5 c. c. extract dilution. On each serum a control should be set up consisting of 0.1 c. c. serum + 0.9 c. c. saline + 0.5 c. c. of 95 per cent. alcohol, diluted 1:6 with saline. Each extract dilution should be controlled by a tube containing 0.5 c. c. extract dilution + 1 c. c. saline.

The tubes are thoroughly shaken and placed in the incubator at 37.5° C. for 20 hours. A preliminary reading may now be made; then the tubes are placed at 14° and 18° C., or in the ice-box for 20 hours, and the final and decisive readings are taken.

The reactions present appearances similar to macroscopic bacterial agglutination or precipitin reactions, and are read with the naked eye, the positives showing varying amounts of precipitates and the negatives remaining opalescent as at the beginning of the test. Sus-

[38] Sachs-Georgi. *Med. Klinik*, No. 33, 1918, 805, and Parker and Haigh, *Archiv. Dermat. and Syphilol.*, 4, 1921, 67.

picious tests are centrifuged at moderate speed for a few minutes, and are proved positive or negative by the fact that in the positives after centrifuging a few definite white compact flocculi can be shaken from the bottom of the tube, whereas the negatives, at most, show a slight grayish sediment which disperses on shaking. The serum controls should remain clear. If a precipitate does occur, the serum is unsuitable and another specimen must be obtained. The extract controls should remain diffusely opalescent, and should show no precipitate.

The Meinecke Reaction.—The Meinecke reaction is one of the many precipitation reactions that have been described of late years for syphilis, since Jacobsthal's observation of a precipitate in the Wassermann reaction. The Meinecke reaction differs from the others in that it adds to the precipitation reaction the question of the solubility of precipitates in salt solution. When certain concentrations of sodium chlorid are added to the ordinary Wassermann antigen, it is flaked out and remains precipitated. Many substances in human serum are prevented from flaking out by the presence of salt, such, for instance, as the globulins, and if flocculated by other means are redissolved by the addition of salt solution. Meinecke [39] claims that if normal and syphilitic sera are precipitated, respectively, with dilute solutions of a lipoidal alcoholic antigen in distilled water, the precipitate formed by the syphilitic serum is less soluble than is that formed from normal sera. *The Kahn Reaction* [39a]—The recent modification of the Sachs-Georgi reaction of Kahn has been found satisfactory by Miss Rockstraw in our laboratory. The essential point in the technique is the antigen preparation which consists in the extraction of dried heart muscle with alcohol, in the ice-box for 9 days and 1 day at room temperature. This avoids the larger lipoid contents of the antigen when incubator extraction is used. This is used both with and without cholesterin, diluted with salt solution. There are many smaller technical points which must be attended to for success, and for these we refer to Kahn's own publications.

COMPLEMENT OR ALEXIN FIXATION AS A METHOD OF DETERMINING THE NATURE OF UNKNOWN PROTEIN

FORENSIC ALEXIN FIXATION TESTS

Our preliminary discussions of the principles underlying alexin or complement fixation have revealed that alexin is bound not only by sensitized cells but also by the specific precipitates formed when an unformed protein antigen is mixed with its specific antiserum. This discovery, made by Gengou, was attributed by him, it will be

[39] Meinecke. *Berl. klin. Woch.*, 54, 1917, 613, also, 55, 1918, 83.
[39a] Kahn, *Archiv. Dermat. and Syphilol.*, 5, 1922, 570, 734, and 6, 1922, 334.

remembered, to the presence of "albuminolysins," or protein sensitizers, antibodies which have been by many observers regarded as separate from the precipitins, but which we believe, for stated reasons (see p. 211), to be very probably identical with the precipitating antibodies or precipitins. However this may be, when a dissolved antigen is mixed with its antiserum alexin fixation is exerted by the complex, and this, even when the reacting quantities, antigen and antibody, are so small that visible precipitation will not take place. For this reason, it is plain, it should be possible by means of complement fixation to detect amounts of a foreign protein too small to be demonstrable by direct precipitation with an antiserum.

The method has, therefore, been suggested chiefly by Neisser and Sachs [40] for the forensic determination of unknown proteins, as an adjuvant to, and improvement upon, the forensic precipitin test. Our discussion of the principles involved in the introductory paragraphs of this chapter will render unnecessary an extensive discussion of the reasoning upon which this reaction is based. It is well to remind the reader, however, of the facts which we have discussed regarding the quantitative proportions which govern the occurrence of precipitation when an antigen, say human serum, is mixed with its antibody, in this case antihuman rabbit serum. The actual precipitation may be absent either when an excess of the antigen is used or when the antigen is present in too small a quantity. Thus a given quantity of the antiserum may precipitate strongly dilutions of the antigen ranging from 1-50 to 1-10,000. No precipitation or, at least, a very slight one only may occur when concentrations stronger than 1-50 are used and when the dilution is greater than 1-10,000. Nevertheless, in both cases, alexin fixation may be exerted by the complex although no precipitation takes place. As Gay [41] has shown, complement fixation may be exerted even when a formed precipitate has been redispersed by the subsequent addition of more antigen. The importance of the forensic reaction of Neisser and Sachs, however, lies chiefly in its application to the detection of quantities of unknown protein too small to be detected by precipitin reactions.

The tests are carried out by mixing a dilution of unknown protein with given quantities of antiserum, adding small quantities of alexin (quantities determined best by previous alexin titration as indicated in our section on the Wassermann reaction); these reagents are left together for a given time at 37.5° C., and then sensitized cells are added to determine whether or not the alexin has been bound.

The table on the following page, taken directly from the article of Neisser and Sachs, *loc. cit.,* will not only illustrate the method of carrying out the reactions but will also give an indication of their extreme delicacy.

[40] Neisser and Sachs. *Berl. klin. Woch.,* Vol. 42, No. 44, 1905, p. 1388.
[41] Gay. Univ. of Cal., "Publications in Pathology," 1912.

Table Taken from Neisser and Sachs, loc. cit., p. 1388

0.1 human antiserum + 0.05 complement and variable amounts of different normal sera (brought to 1 c. c. volume with salt solution); the mixtures kept 1 hour at room temperature. Then added 1 c. c. 5 per cent. washed beef blood + 0.0015 c. c. amboceptor and left 1–2 hours at 37° C.

The results are as follows:

<table>
<tr><th rowspan="2">Amounts of normal serum</th><th colspan="8">Hemolysis on addition of serum of:</th></tr>
<tr><th>Man</th><th>Monkey</th><th>Rat</th><th>Pig</th><th>Goat</th><th>Rabbit</th><th>Ox</th><th>Horse</th></tr>
<tr><td>0.01</td><td>0</td><td>0</td><td rowspan="6">complete</td><td rowspan="6">complete</td><td rowspan="6">complete</td><td rowspan="6">complete</td><td rowspan="6">complete</td><td rowspan="6">complete</td></tr>
<tr><td>0.001</td><td>0</td><td>0</td></tr>
<tr><td>0.0001</td><td>0</td><td>moderate</td></tr>
<tr><td>0.00001</td><td>slight</td><td>complete</td></tr>
<tr><td>0.000001</td><td>complete</td><td>complete</td></tr>
<tr><td>0</td><td>complete</td><td>complete</td></tr>
</table>

It will be seen that 0.00001 c. c. of the normal human serum still gave almost complete complement fixation of 0.05 c. c. of complement in the presence of 0.1 c. c. of the antihuman serum. The table also shows that this reaction follows a general law of relative specificity so often noted in other reactions, namely that, of all the animals tested, the serum of monkeys alone gave reactions with the human antiserum; and this in quantities as small as 0.001 cubic centimeter.

The forensic complement fixation reaction of Neisser and Sachs is both theoretically and practically valid. Its extensive use in many investigations for theoretical purposes has well established its reliability. However, it is more complicated and requires much more experimental training and care than does the simpler precipitin test, and it will rarely occur that an unknown protein is available in quantities too small to permit of successful precipitation.

THE USE OF COMPLEMENT FIXATION TESTS IN THE DIAGNOSIS OF MALIGNANT NEOPLASMS

A great many attempts have been made to establish a method of complement fixation by which a diagnosis of malignant tumors could be made. It had been hoped that the substance of malignant tumors might contain a form of protein or protein lipoid combination which might represent substances specific for such tumors, and might therefore functionate as a specific antigen. On this basis it might be possible that the serum of tumor patients would contain a specific antibody which could react with a specific antigen in tumor extracts, with the resulting formation of an alexin-fixing complex.

No experimental facts have so far justified our assumption of the presence of either specific antigen in tumor extracts, or that of a specific antibody in the serum of such patients. However, we have seen that the Wassermann reaction is a perfectly useful clinically diagnostic method, in spite of

the fact that the antigen need not be specific, and the purely empirical basis on which the syphilis reaction is at present based has justified extensive attempts to establish an analogous empirical method for tumor diagnosis.

The literature on this question is confusing. A number of observers using antigens variously prepared from tumor substances have reported favorable results. Simon and Thomas [42] report many positive reactions, as do Sanpietro and Tesa [43] and a number of others. Clowes [44] has carried out a reaction on sarcoma rats and obtained positive reactions in animals in which the tumors were small, negative ones when the tumor had grown to a large size. Ranzi, on the other hand, obtained negative results throughout. Ranzi [45] found that normal serum would often give complement fixation with carcinoma extracts, also that many tumor extracts and sera of tumor patients inhibited complement by themselves. The reactions were so irregular that he assumed them to be without value. Recently the subject has been very thoroughly investigated by v. Dungern.[46]

Von Dungern claims to have finally evolved a method by which the diagnosis of malignant disease can be made with reasonable accuracy. Like the Wassermann reaction his method is purely empirical. He admits that probably it is not a specific antibody determination and depends rather upon the presence of pathological products of metabolism in the sera of tumor patients. The reliability of his method depends upon the observation of a number of details which he has determined empirically.

He obtains his antigen in a purely non-specific manner, using, as just stated, for this reaction acetone extracts of human blood cells. We take the description of the reaction entirely from his own article in "Weichhardt's Jahresbericht." The antigen is prepared in the following way: Blood is taken from a vein, preferably from a paralytic patient, since v. Dungern claims that individual specimens of blood vary, and he has had the best results with that of paralytic cases. Clotting is prevented by sodium oxalate and the blood cells are thoroughly washed in the centrifuge. To the sediment are added 19 volumes of pure acetone (Merck). This is allowed to stand three days at room temperature and is occasionally shaken during this time. It is then filtered, the acetone evaporated in the incubator at 37° C., and the residue taken up in 96 per cent. alcohol. This alcoholic extract is diluted before use with four parts of salt solution. Of this final preparation 0.8 c. c. is used in the individual test.

Particular precautions must also be taken in the handling of the serum of the patient. In his earliest tests v. Dungern determined that the inactivation of the tumor sera greatly diminishes their specific fixation properties, and for this reason he at first advised that the serum be used unheated. He has found recently that the best results are obtained when the serum is heated to 54° C., together with a little sodium hydrate solution. He handles the blood in the following way: After being taken from the patient it is allowed to stand 1 to 2 days in the refrigerator; just before use he adds two parts of an $\frac{n}{50}$ NaOH solution with one part of serum and heats it for half an hour at 54° C. As it is important that the sodium hydrate should contain no sodium carbonate, he advises the use of the Kahlbaum preparation. In setting up the test he uses graded quantities of the mixture corresponding to

[42] Simon and Thomas. *Jour. Exp. Med.*, Vol. 10, 1908.

[43] Sanpietro and Tesa. Cited from v. Dungern in "Weichhardt's Jahresbericht," etc., Vol. 8, 1912, p. 163.

[44] Clowes. *Jour. A. M. A.*, 1909, Vol. 52.

[45] Ranzi. *Wien. klin. Woch.*, 1906, p. 1552.

[46] V. Dungern. *Münch. med. Woch.*, Nos. 2, 20, 52, 1912; *Berl. klin. Woch.*, 1913, "Weichhardt's Jahresbericht," Vol. 8, 1912, p. 163.

0.2, 0.1, 0.05, and 0.025 c. c. of the original serum. To each of these quantities he adds the stated quantity, 0.8 c. c. antigen preparation described above, and the 0.05 guinea pig complement. Controls must be set up with the antigen alone and with the patient's serum alone to prevent error from independent fixation by these substances. These reactions are allowed to stand three hours at room temperature, and then one cubic centimeter of a 5 per cent. solution of beef blood sensitized with two units of hemolytic serum is added (as in the Wassermann reaction). It is important to use a strongly sensitizing serum, so that not too much of the hemolytic rabbit serum must be added to the tubes. Experiments done in this way with normal sera usually result in complete hemolysis within one hour, although in certain other diseases, i. e., tuberculosis and syphilis, slight inhibition may result. However, fixation with the patient's serum in quantities of 0.1 c. c. or less is, according to von Dungern, fairly specific for malignant tumors, since normal sera treated in the way described usually do not cause fixation in quantities of less than 0.2 c. c. and, in syphilis and tuberculosis, if fixation is at all present, it is usually not evident in quantities less than 0.1 c. c.

With a reaction so carried out von Dungern has examined 244 cases. The following tabulation states his results:

Malignant tumor of	No. of cases	Reaction positive*
Pharynx	3	3
Esophagus	6	6
Stomach	15	11
Rectum	14	10
Larynx	2	2
Tongue	5	5
Bladder	1	1
Breast	22	22
Uterus	10	10
Skin	8	7
Ethmoid bone	1	1
Upper maxilla	1	1

* Taken from von Dungern, "Weichhardt's Jahresbericht," Vol. 8, 1912, p. 174.

We report von Dungern's results exactly as he states them in his last summary, since his well-known experimental ability necessitates serious consideration of all of his work. We may say, however, that a survey of the entire literature of complement fixation in the diagnosis of malignant tumors does not yet justify our acceptance of this method as of anything like the established value which the similar method has attained in syphilis.

Complement Fixation in Glanders

The diagnosis of glanders by the mallein test and by agglutination has been recently reënforced by the method of complement fixation. In carrying out these tests the method of preparation of the antigen is of the greatest importance. The directions which we give are those employed in the Diagnostic Laboratory of the New York Department of Health, under the immediate supervision of Dr. McNeil and Miss Olmstead, from whom we have our information.

The particular strain of glanders bacilli employed seems to be of little importance. The organisms are grown on 1.6 per cent. acid glycerin-potato-agar. This stock culture is transplanted every other day. From it cultures are planted upon salt-free veal peptone agar. It is of the greatest importance that this medium shall be neutral to phenolphthalein. After twenty-four hours in the incubator the growth is washed off with distilled water, which also should be neutral, and the emulsion heated for from four to six hours at 80° C. in a water bath. It is then filtered through a Buchner filter simply to facilitate subsequent filtration through a Berkefeld "N" or "V" filter. After filtration this antigen is again sterilized for one hour at 80° C. and then on two successive days at 56° C. for one-half hour.

The fixation tests carried out with these antigens have yielded excellent results as reported by Dr. McNeil[47] at the New York Serological Society.

It is unnecessary to give further directions as to the technique of this reaction, since it is simply that of complement fixations in general, the chief difficulty being that of antigen preparation.

Complement Fixation in Gonorrheal Infections

There are certain conditions following gonococcus infection of the genito-urinary tract which are not easily distinguished from a number of other tests unless the organisms can be cultivated or a specific serum reaction can be applied. Most important of these are gonorrheal rheumatism, salpingitis, and endocarditis. Complement fixation with the sera of such patients, and an antigen produced from gonococci, has been employed by many observers during recent years, and promises to be of great value.

Here, too, the production of the antigen is the only feature of the reaction which has offered difficulties. Since the researches of Torrey have shown that not all races of gonococcus are antigenically alike, it seems necessary to produce a polyvalent antigen. At the New York Department of Health at present the antigen is prepared by using the ten Torrey strains. Stock cultures are carried on neutral veal agar and cultures are planted upon salt-free veal agar. Twenty-four-hour growths, washed off in neutral distilled water, are kept in a water bath at 56° C. for two hours, and then filtered through, first, a Buchner and then a Berkefeld filter. They are then sterilized for one hour.

Complement Fixation in Tuberculosis

Attempts to apply complement fixation to the diagnosis of tuberculosis have been made by as many as thirty or more investigators

[47] McNeil, Archibald. N. Y. Serological Soc. Meeting, April 4, 1914.

with varying results. Wassermann and his collaborators attempted it before they succeeded in developing the Wassermann reaction in syphilis. Recently, intensive work has been done on the subject by Besredka,[48] Petroff,[49] Craig,[50] Bronfenbrenner,[51] and Miller and Zinsser.[52] Results have warranted the application of the reaction to clinical tuberculosis, although the actual degree of usefulness of the reaction must still await the multiplication of cases tested. The difficulty has of course consisted in the development of a suitable antigen. The antigen of Petroff has consisted of a filtrate of potato broth. Besredka has used a filtrate of cultures made upon egg broth. Craig has used Besredka's antigen and suspensions of ground bacilli. Miller and Zinsser [52] have employed an antigen made by triturating living and dead bacilli with crystals of table salt, then adding distilled water to isotonicity. With all of the antigens favorable results have been obtained. Our own results seem to check up with clinical diagnoses in over 80 per cent. of the cases and so far have appeared to give practically no positive results in negative cases and indicated only active tuberculosis and not healed lesions.

The reactions are carried out by methods entirely analogous to those employed in the Wassermann reaction, but careful antigen titrations must be done.

The Preparation of Bacterial Antigens for Alexin Fixation Reactions.—We have mentioned only a few of the more commonly used alexin fixation reactions with bacteria. Alexin fixation can be used for experimental and diagnostic purposes in the case of almost any species of bacteria. The technical difficulties have involved chiefly the production of a suitable antigen, since many bacterial suspensions and extracts are non-specifically anticomplementary, or, in other words, these bacterial preparations fix alexin by themselves without previous sensitization. The reactions, for this reason, have necessitated a very careful titration of the maximum amounts of alexin fixed by the unsensitized antigen in order that this might be allowed for in the final reaction. Such a procedure, when the anticomplementary action of the suspension is at all marked, involves delicate manipulation and titrations, and great care in interpretation.

We have recently made studies of bacterial antigens which are mentioned in other parts of this book; but it may be useful at this point to call attention to the practical advantages for all kinds of bacterial alexin fixations represented in the use of our so-called

48 Besredka. *Zeitschr. f. Immunit.*, 1914.

49 Petroff.

50 Craig. *Am. Jour. Med. Sc.*, Dec., 1915, p. 781.

51 Bronfenbrenner. *Arch. Int. Med.*, 1914, No. 6, 786. *Zeitschr. f. Immunit.*, 1914, XXIII, 2, 21.

52 Miller and Zinsser. *Proc. Soc. Exper. Biol. and Med.*, 1916, 134. *Jour. Lab. and Clinical Med.*, Vol. I, p. 817.

residue antigens. These substances which are antigenic in the sense that they unite with antibody, giving both clear precipitations and alexin fixations with specific sera and almost entirely devoid of anti-complementary action, consist in the residue left when alkalin extracts of bacteria are boiled for a short time with acid, filtered clear and neutralized. The antigens may be further purified by precipitation in the neutral residues with ten volumes of alcohol, and resolution in normal salt solution. The technique for the preparation of these antigens from different bacteria varies.

Tubercle Bacilli.—This antigen devised by us [52a] is being used extensively by Petroff at the present time. The result of his investigations has not yet been published. In the case of tubercle bacilli, the antigens are made by shaking pulverized tubercle bacilli, about 50 milligrams in 100 c. c. of slightly alkaline salt solution. After about three hours' shaking, a massive precipitate is obtained by acidifying with acetic acid, added drop by drop. This is filtered off and the filtrate boiled for from 2 to 5 minutes over a free flame, still in the acid condition. The liquid is allowed to cool and the slight cloud which forms, is filtered off by passing through a Berkfeld. The filtrate is neutralized to about Ph 7, and the precipitate of phosphates which forms as the neutral point is approached, again removed by filtration. The water-clear residue which gives a slight precipitation when mixed with about 10 volumes of alcohol can be used directly, and titrated for alexin fixations without further manipulation, or may be precipitated with 10 volumes of alcohol and taken up in any desired concentration with sterile salt solution. In this form it can be kept a long time and can be boiled repeatedly without losing its antigenic properties.

Influenza Bacilli.[52b]—Influenza bacilli are grown on chocolate agar in large pie plates, or any moderately large surface, washed off with salt solution and shaken in neutral salt solution for about two hours, then filtered and treated as above. The influenza bacillus antigen is much less stable than the tubercle bacillus preparation.

Pneumococci.—Pneumococci are washed off agar surfaces, shaken a short time, not more than an hour is necessary, in slightly alkaline salt solution, acidified, boiled, etc., and in other ways treated as described for the tubercle bacillus above. The pneumococcus antigen is extremely stable.

Meningococcus.—Meningococcus antigen is prepared just exactly as for pneumococci, only in this case, as in the influenza bacillus, great care must be taken to preserve the final residue in a slightly acid condition and to apply no heat except when the antigen is slightly acid. If alkaline boiling weakens and eventually destroys it.

Staphylococci.—Staphylococcus residue antigen may be pre-

[52a] Zinsser. *Jour. Exp. Med.,* Vol. 34, 1921, p. 495.
[52b] Zinsser and Parker. *Jour. Exp. Med.,* Vol. 37, 1923, p. 275.

pared in the same way, except that with the staphylococci it is necessary to collect dried bacteria, grind them and extract the pulverized staphylococci as the tubercle bacillus. They are subsequently treated like tubercle bacillus powder.

The only organisms, so far worked with, with which we have not succeeded in obtaining satisfactory amounts of residue antigen are typhoid bacilli.

CHAPTER IX

THE PHENOMENON OF AGGLUTINATION

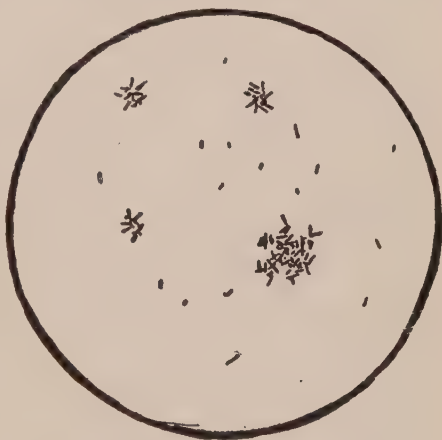
When bacteria are added to homologous immune serum there occurs a peculiar agglomeration of the individual micro-organisms into small clumps. The phenomenon is so general and so easily observed that it is not surprising that it was noticed and reported by a number of workers during the period of active investigation upon serum reactions which preceded and followed the discovery of the Pfeiffer phenomenon. Thus, in the years from 1891 to 1895, Metchnikoff,[1] Charrin and Roger,[2] Isaeff and Ivanoff,[3] Washburn,[4] and several other workers made this observation with a variety of bacteria and immune sera. But all of these observers failed to follow up or analyze the process they incidentally noticed in the course of other investigations. A thorough study of the phenomenon was not made until 1896, when Gruber and Durham,[5] in Vienna, in the course of their studies upon bacteriolytic reactions with colon bacilli and cholera spirilla, again noticed the agglutination of these bacteria in homologous immune sera, recognized the specificity of the reaction, and called attention to its apparent independence of other previously studied serum phenomena.

The process known as agglutination consists in the following train of occurrences. If we add to an even emulsion of bacteria a small amount of homologous immune serum the micro-organisms may be seen to collect rapidly in groups or masses, with a resultant clearing of the fluid in which they have been suspended. The clumps of bacteria gather in flakes which, not unlike flakes of snow, sink to the bottom of the test tube. The speed and completeness with which this phenomenon occurs depend, as we shall see, upon the agglutinating strength and other qualities of the serum which is employed, but the essential process of clumping is alike in all cases.

There are a large number of different methods by which this occurrence can be observed, each one particularly adapted to some special purpose for which the reaction is carried out. Gruber and Durham, who were investigating the properties of bacteriolysins

[1] Metchnikoff. *Ann. de l'Inst. Past.*, 1892.
[2] Charrin and Roger. *C. R. de la Soc. de Biol.*, 1889.
[3] Isaeff and Ivanoff. *Zeitschr. f. Hyg.*, Vol. 17, 1894.
[4] Washburn. *Jour. Path. and Bact.*, 1896, p. 228.
[5] Gruber and Durham. *Münch. med. Woch.*, 1896,

when they observed agglutination, naturally recognized the specific nature of the reaction and proposed to make use of it for the purpose of bacterial differentiation and species determination. For this purpose, which has become one of the most important of the practical applications of the agglutination reaction, the phenomenon is best observed by the so-called "macroscopic method," in which a series of serum dilutions are mixed, in small test tubes, with equal volumes of emulsions of the bacteria. Thus, if we wish to determine the nature of an unknown bacillus, suspected of belonging to the typhoid bacillus group, by this method, we may determine its agglutination in the serum of an animal immunized with a known strain of typhoid. The tubes are incubated after the mixtures have been made, and the agglutination which has taken place in the various tubes is recognized by a clearing up of the fluid and the flaking of the bacteria after from one to three hours. The test tube method has the advantage of permitting the use of larger quantities of reagents than can be used in the other methods described below, and therefore more exact quantitative measurements can be made.



MICROSCOPIC AGGLUTINATION.

Although this method for the determination of bacteria has found universal application, it is probably most frequently employed at the present time for the rapid identification of colonies of doubtful typhoid or dysentery, incident to the isolation of these organisms for stools by such methods of plating as those of Conradi-Drigalski, of Endo, or of Hiss. The suspicious colonies can thus be fished directly to an agar slant, and the cultures, when developed, emulsified and identified by agglutination. The advantages of such a method for the determination of the biological interrelationship of the organisms of a given group, like, for instance, that of the dysentery bacilli, are obvious.

An ingenious use of this reaction was also made by Shiga when he determined, among various bacteria isolated from the stools of dysentery cases, the particular one which was specifically agglutinated by the patient's serum, thus discovering the dysentery bacillus which bears his name.

Within a few months after the publication of Gruber and Dur-

ham's work, Widal and, apparently independently of him, Grünbaum,[6] by a process of reasoning the converse of that detailed above, applied the reaction to the diagnosis of infectious disease.

It is obvious that a human being or an animal infected with a given variety of bacteria may develop agglutinating properties against these bacteria. It is of great value, therefore, to determine the agglutinating power of the serum of a patient for the bacteria which are known to cause the disease suspected in the particular case in which a diagnosis is desired. This method has become a routine measure in the early diagnosis of typhoid fever under the name of "Widal" or "Gruber-Widal" reaction and, since the quantities of serum which can easily be obtained from a patient are usually small, it is convenient to carry out the reaction by the microscopic method. This consists in mixing serum and bacterial emulsion in hang-drop preparations and observing them with the microscope. An excellent method, also, is the so-called Proescher [7] method in which serum and bacterial emulsion are mixed in small watch-glasses or salt cellars. Proescher used this method extensively in the study of staphylococcus agglutinations. The mixtures in the salt cellars were set away at 37° C. for two hours, and then observed with a magnification of 60 to 70 diameters.

Close observation of the occurrence under the higher power of a microscope shows that the bacteria, if motile, lose their motility, if non-motile the Brownian motion is arrested. They are then rapidly gathered in small clumps, isolated individuals between these clumps being gradually drawn into them, until finally the fluid between the masses is entirely clear. This complete clearing, of course, happens only when there is not an excess of bacteria, for, like other serum reactions, this phenomenon is a quantitative one in which definite proportions must be maintained.

Clinically the most frequent use of the agglutination reaction is in the diagnosis of typhoid fever. The technique used for this test is, in the large majority of cases, the microscopic hang-drop method. In Germany the Proescher method is sometimes used, and the microscopic method with dead organisms, as first introduced by Ficker, is also not uncommon at the present day.

Since the serum of normal human beings very often contains moderate agglutinating powers for the typhoid bacillus, the diagnostic value of the reaction in this disease depends upon the elimination of this error by sufficient dilution. If dilutions of the serum of from 1-40 to 1-60 are used diagnostic errors on this point are avoided, since the normal agglutinating power of human beings is never such that typhoid bacilli will be clumped by it in these dilutions within one hour. Prompt clumping in serum dilutions of 1-20

[6] Grünbaum. *Lancet*, 1896, Vol. 2.
[7] Proescher. *Centralbl. f. Bakt.*, Vol. 34, 1903.

is fairly reliable, but does not absolutely exclude an unusually high normal agglutinating power. In carrying out tests clinically dilutions of 1-20, 1-40, and 1-80 are usually made and observed for one hour. From such tests diagnosis can be made without danger of error. In rare cases of icterus the agglutinating power for typhoid bacilli may be increased. Just what is the cause of this is not certain; Wood [8] reports cases in which agglutination of 1-40 was present with slight jaundice (hepatic cirrhosis). On the other hand he has frequently failed to notice agglutination in other cases of intense jaundice. It is not impossible, as Wood suggests, that the occasional presence of unusual agglutinating power in individuals with jaundice has some relation to the frequent persistence of typhoid bacilli in the gall bladder.

Occasionally it will be noticed that dilutions of the patient's serum of 1-5 to 1-20 fail to agglutinate, while higher dilutions will give positive tests. This is referable to the so-called "pro-agglutinoid zone," the principles underlying which we shall discuss in another place.

The Widal test in typhoid cases rarely appears before the end of the first week, and, in the majority of cases, is present before the end of the second week. It may proceed for months, although Wood states that he has seen it disappear at the end of three to six weeks.

In *paratyphoid fever* the diagnosis can often be made by agglutination, and in *dysentery,* as we have seen, the fact that the patient's blood agglutinated the bacteria was one of the important facts utilized by Shiga in his discovery of the organism which bears his name.

In *pneumonia* agglutination of the pneumococcus, isolated from the patient's sputum by sera prepared by immunization with various types of pneumococci, has become of considerable importance clinically, since Neufeld and Haendel and, in this country, Cole, Dochez, and Gillespie have determined that there are a number of different types of this micro-organism. The use of pneumococcus serum in the disease will be of value only if a serum is used which has been produced with an organism of the same type as the one infecting the patient. Therefore, determinations of the type by highly potent agglutinating sera give a finger-point to the variety of serum to be used. Whatever may be the eventual outcome of the serum treatment in pneumonia, no results whatever can be expected, according to our present knowledge, unless such determinations are made. The technique of agglutinations in pneumococcus work is facilitated by growing mass cultures of organisms, as advised by Hiss, in flasks of glucose broth containing 1 per cent. calcium carbonate, but ordinarily no difficulty is encountered and no special methods are necessary.

[8] Wood. "Chemical and Microscopical Diagnosis," D. Appleton & Co., p. 242.

The same method of growing micro-organisms is useful in the case of streptococcus agglutinations, since the insoluble calcium carbonate, if thoroughly shaken, breaks the chains of streptococci and thereby facilitates judgment as to the reaction.

Agglutination reactions have been of considerable usefulness also in the diagnosis of glanders in horses. The early work on this subject was done chiefly by Macfadyean,[9] and the reaction has been particularly studied by Wladimiroff.[10] Since the serum of normal horses will often agglutinate glanders bacilli in dilutions of as much as 1-500, Wladimiroff advises making the positive diagnosis on dilutions only higher than 1-1,000, since he states that normal horses may occasionally reach an agglutination titre of 1-1,000. The same writer states, moreover, that glanders bacilli are subject to great variations in agglutinability, and that for this reason the choice of a suitable strain is of great importance.

The motility of bacteria has absolutely no relation to the reaction, and their agglutination is entirely passive.

Some of the earlier investigators of agglutination associated the reaction with alteration in the flagellar mechanism of the micro-organisms. It is now well known, however, that non-motile, as well as motile, bacteria are subject to the phenomenon, and that no visible change in the appearance or arrangement of flagella accompanies the clumping. Although this is the case, observation of the motility of such organisms as the bacillus of typhoid fever, while subjected to the action of agglutinating serum, may be of great value in aiding in the determination of the degree of completeness with which the reaction is taking place.

Agglutination, furthermore, does not lead to the death of the bacteria. Of course, whenever the reaction is carried out in unheated serum the concomitant effects of the bactericidal substances bring about bacterial death. Agglutination does not, however, depend upon the coöperation of alexin, and serum may be inactivated without interference with its power of agglutination. In such heated serum clumping takes place without bactericidal effects, and, more than this, the bacteria may grow, if exposed to proper temperature conditions, when suspended in the serum. In fact, it is of considerable interest to carry out the reaction in this way, for the bacteria growing in agglutinating serum form long convoluted threads and skeins even when in ordinary culture they habitually occur as separate individuals. Thus colon bacilli, typhoid bacilli, pneumococci, cholera spirilla, and other organisms, which ordinarily grow as free single cells, or, at most, in chains of two or three, if kept in the incubator for ten to twelve hours together with homologous serum, will grow in long, delicate chains, like those of streptococci. This

[9] Macfadyean. *Jour. Comparative Path. and Ther.*, Vol. 9, 1896.
[10] Wladimiroff. "Kolle u. Wassermann Handbuch," 2d Ed., Vol. 5.

form of reaction has been especially studied by Pfaundler,[11] who attributed particularly delicate specificity to it. However, the "Thread Reaction," as it is sometimes called, may be regarded as merely another manifestation of the phenomenon of agglutination and subject to the same laws and limitations of specificity which apply to other methods.

The purely passive rôle played by the bacteria in agglutination is best shown by the fact that dead bacteria, killed in various ways, are specifically clumped just as are the living cultures.[12] On this fact depends the method spoken of as "Ficker's Reaction," in which emulsions of typhoid bacilli, killed by formaldehyd or carbolic acid (distributed commercially), are agglutinated in small test tubes by the serum of typhoid patients. The original method of Ficker is said to be a proprietary secret; however, a number of other methods which attain the same purpose are in use in various places. Volk [13] describes the method used in Vienna, and states that there carbolic acid is used to kill the cultures. Similar to this is the method described by J. H. Borden,[14] who proceeds as follows:

The bacilli are grown on agar slants in large tubes for 24 hours. They are then washed from the medium with a sterile mixture of salt solution 450 parts, glycerin 50 parts, and 95 per cent. carbolic acid 2.5 parts. After this solution has been kept a week it becomes translucent and by this time the bacilli are dead. The preparation is then ready for use and can be kept a long time in dark bottles in a cool place. Borden very carefully controlled this bacterial emulsion with positive and negative typhoid sera and found it reliable. The great advantage of all these methods, of course, consists in the possibility of furnishing the general practitioner with materials for clinical agglutination tests in which the necessity of preserving and suspending living cultures is eliminated.

The facts which we have just considered tend to show that agglutination is not a vital phenomenon [15] dependent in any way upon the living nature of the bacterial cell, but, like other phenomena of antigen-antibody reactions, a purely chemical or physical process in which the substance of the bacterial cell enters specifically into relation with the agglutinating factor of the serum. In uniformity with other analogous reactions the antigenic substance is here spoken of as "agglutinogen," the antibody as "agglutinin."

Agglutinogen.—The agglutinogen, or agglutinin-inducing substance in the bacteria is apparently an inherent part of the bacterial protein, and agglutinins may be produced in animals by injection

[11] Pfaundler. *Wien. klin. Woch.*, 1898, and *Centralbl. f. Bakt.*, I, Vol. 23, 1898.

[12] Bordet. *Ann. Past.*, Vol. 10, 1896.
[13] Volk. "Kraus und Levaditi Handbuch," Vol. 2.
[14] Borden. *Medical News*, N. Y., Mar., 1903.
[15] Bordet. *Ann. Past.*, Vol. 10, 1896.

not only of living and dead whole bacteria, but by bacterial extracts, prepared in various ways. And, furthermore, just as the agglutinins of serum are absorbed out of a serum by the whole bacteria, they may be neutralized by the dissolved bacterial extracts.

Just what the nature of the agglutinogen is has been a matter of prolonged controversy, Pick [16] and others claiming that it is possible to obtain an agglutinogen by alcohol precipitation from old bacterial cultures which, upon further purification, can be found to give none of the usual protein reactions (Biuret, Millon). It is by no means certain, however, that Pick's results are correct. That the agglutinogen is, to a certain extent, subject to dialysis has been claimed because of experiments in which specific agglutinins have appeared in the sera of animals into whose peritoneal cavities celloidin sacs, filled with bacteria, have been placed.[17]

There has been a great deal of discussion regarding the possible localization of the agglutinogen of bacteria in the ectoplasmic layers of the cells, and especially in the flagellar substance. We have seen that, as a matter of fact, nonmotile bacteria are subject to the phenomena of agglutination just as are the motile forms, but numerous attempts were made during the earlier stages of our knowledge of this reaction to demonstrate that changes in ectoplasm and flagella accompanied the actual agglutination. Gruber [18] himself held the opinion for a time that the clumping was due to an ectoplasmic swelling which rendered the bacteria sticky, causing them to hold together after chance approximation. He soon gave up this idea himself, but a similar theory was for some time upheld by Nicolle [19] and others.

Malvoz [20] in 1897 devised an ingenious method by which he believed that he could show that the agglutination of bacteria depended upon their ectoplasmic substances. He passed the typhoid emulsion through Chamberland filters and, when the bacilli had been caught upon the filters, he subjected them to prolonged washing. The bacilli, now removed from the filter by passing fluid through in the opposite direction, were no longer motile or agglutinable either by formalin, safranin, or other chemical agents, nor by agglutinating sera. Dineur,[21] repeating the experiments of Malvoz, came to the same conclusions. He decided that in agglutination the bacteria became entangled with each other by means of the flagella. Harrison,[22] in later studies working under Tavel, attempted to dissolve out the ectoplasmic layers of bacteria with pyocyanase, and from his experiments also came to the conclusion that the agglutinogen was contained in the external layers. Similar results were obtained by De Rossi.[23]

[16] Pick. "Hofmeister's Beiträge," 1901, 1902.

[17] This would be in keeping with Pick's work just referred to, and should be subjected to the same criticism before final acceptance. For a more detailed discussion of these conditions the reader is referred to the article by Paltauf, "Kolle u. Wassermann Handbuch," Vol. 4, part 1.

[18] Gruber. *Münch. med. Woch.*, 1896.

[19] Nicolle. *Ann. de l'Inst. Past.*, 1898.

[20] Malvoz. *Ann. de l'Inst. Past.*, Vol. 11, 1897.

[21] Dineur. *Bull. de l'Acad. de Méd. de Belge,* 1898, cited from Smith and Reagh.

[22] Harrison. *Centralbl. f. Bakt.*, Vol. 30, I, Orig. 1901.

[23] De Rossi. *Centralbl. f. Bakt.*, I, Vols. 36 and 40.

Further studies on the same problem are those of Smith and Reagh.[24] These investigators worked with two strains of bacilli, both of which they regarded as belonging to the hog-cholera group, though the one was motile and the other nonmotile. When rabbits were immunized with the nonmotile bacillus an agglutinin was obtained which acted upon this bacillus differently and less powerfully than did the agglutinin produced with the motile one. Contact with the nonmotile bacillus did not deprive the serum produced with the flagellated organism of the agglutinins for the latter. They conclude that two agglutinins were involved—one incited by the ectoplasm and flagellar substance, the other by the bacterial cell body proper. Rehns as well as Paltauf have criticized these results by questioning the species identity of the two bacilli employed in the experiments, referring the phenomenon to the occurrence of group agglutination.

As a matter of fact our present knowledge of agglutination no longer justifies the association of agglutination with flagella. Nonmotile as well as motile bacteria are readily agglutinated, and we have much evidence which will be discussed presently which shows that the agglutination reaction is governed by many of the laws which obtain in colloidal flocculations. This, however, does not exclude the possibility that it is the ectoplasmic zone chiefly which takes part in the reaction. Furthermore, loss of motility, which always accompanies agglutination when a motile organism is under observation, is an extremely valuable aid in guiding us in our judgment of incomplete reactions.

That changes may be brought about in bacterial agglutinogen by various methods of treatment has been shown by a number of workers, although the fundamental principles underlying such changes are not at all clear.

Joos [25] was the first to study agglutination with particular reference to the effects upon the reaction of heating both the antigen and the antibody. On the basis of extensive and complicated experiments upon the agglutinin produced in horses by immunization with heated and unheated typhoid bacilli, he drew the conclusion that agglutinogen (agglutinin-inducing substance) in bacteria was not a single element but consisted of at least two definite parts of which he speaks as *a* and *B*-agglutinogen. *a*-agglutinogen is a constituent of the living bacteria, and is easily destroyed at 60° to 62° C. The *B*-agglutinogen is also present in normal bacilli, but is more heat-resistant and will withstand 60° to 62° C. for several hours. The injection of living unheated bacilli then induces the formation of both *a* and *B*-agglutinin, which have respectively a particular affinity for *a* and *B*-agglutinogens. The injection of heated bacilli, on the other hand, induces the formation only of *B*-agglutinin and not a trace of *a*-agglutinin. The *a*-agglutinin is considerably heat-resistant, resisting 60° to 62° C., whereas the *B*-agglutinin loses its agglutinating property

[24] Smith and Reagh. *Jour. Med. Res.*, Vol. 10, 1903.
[25] Joos. *Centralbl. f. Bakt.*, Vol. 33, 1903.

when heated to 60° C. The *a*-agglutinin is entirely incapable of uniting with *B*-agglutinogen. However, *B*-agglutinogen can combine or react with both the *a* and *B* constituents of the bacilli. For this reason Paltauf has spoken of agglutinin produced with the heated bacteria as "umfänglicher." This is a point of great interest, and if Joos is right is, of course, of considerable practical importance.

However one may look upon these experiments, as well as the similar ones of other workers, it seems established that heating bacteria leaves them still capable of inciting agglutinins powerfully and rapidly, perhaps of an "umfänglicher" nature than those produced with the native cells.

Heating bacteria may also alter their agglutinability. Thus, according to Eisenberg and Volk,[26] heated above 65° C. the bacteria no longer agglutinate in the presence of specific immune serum, but still absorb agglutinin. Eisenberg and Volk, therefore, distinguished between a heat-sensitive constituent of the antigen, which is particularly associated with the clumping, whereas the thermostable substance represents the haptophore or combining portion. It seems simpler, in this case also, to assume a change in the colloidal stability of the bacteria by heating than to seek it in a differentiation into combining and agglutinable parts of the same antigen.

The points raised by Joos' work have been followed up particularly by Kraus and Joachim [27] and by Scheller.[28] Scheller summarizes the results of his work as follows: First, in agreement with Joos he found that immune sera obtained by injection of bacteria modified by heat vary considerably from each other. Secondly, immunization with living typhoid bacilli produces sera which agglutinate living bacilli very highly and less highly bacilli heated to 60° C. The titre of agglutinating serum is altered very little toward living bacilli after heating to 60° to 62° C., but is sometimes diminished toward bacteria that have been heated. Bacilli that have been heated to 100° C. but slightly agglutinate unheated serum. Sera produced by the injection of typhoid bacilli heated to 60° to 62° C. agglutinate with both living and heated bacilli. Very important furthermore in Scheller's work are the determinations that typhoid bacilli which have been heated absorb agglutinins out of the sera more actively than do the unheated bacteria, and that the highest agglutinin titres can be obtained by agglutination with bacilli that have been heated to 60° C. The analogy of Scheller's results with similar work done in connection with the precipitin reaction is striking and will be referred to in another place.

Alterations in the agglutinability of bacteria may also occur spontaneously, without previous heating, as in the preceding experi-

[26] Eisenberg and Volk. *Zeitschr. f. Hyg.*, Vol. 40, 1902.
[27] Kraus and Joachim. *Centralbl. f. Bakt.*, I, Vol. 36, 1904.
[28] Scheller. *Centralbl. f. Bakt.*, Vol. 36, 1904, and Vol. 38, 1905.

ments. It has been frequently noticed that typhoid bacilli, recently cultivated out of the human body, will not agglutinate in sera which have high agglutinating power for strains kept for some time on laboratory media. Much investigation has been focused upon the determination of the cause for this, and although many explanations have been suggested no satisfactory solution has been found. Most workers who have attempted to attack this problem have based their reasoning upon the receptor conception of Ehrlich and have assumed that such inagglutinable bacteria are characterized by a diminished equipment in "receptors." Such strains have been especially well studied in the case of typhoid bacilli and cholera spirilla. Inagglutinable typhoid bacilli have been cultivated by many investigators from the spleen, gall bladder, and urine of typhoid patients, and many of these, when studied for prolonged periods, have been found to regain normal agglutinability after several generations of cultivation upon artificial media. Apparently some alteration of the bacilli had taken place in the presence of the body fluids (immune serum) which affected their sensitiveness to the agglutinins, i. e. their ability to unite with or absorb this antibody. The phenomenon involves an important principle, emphasized some years ago by Professor Welch, namely, that the bacteria may acquire a quasi-immunity against the attacking forces of the body, a property which may be responsible for the increase of virulence noted when some bacteria are repeatedly passed through the bodies of animals, and, indeed, alterations of virulence signify biologically a process of adaptation on the part of the bacteria just as increased immunity indicates a similar process on the part of the invaded subject.

This lessened susceptibility to antibodies is noticeable not only in strains cultivated from the body in disease, but can be produced artificially by cultivating the bacteria in inactivated homologous immune serum. This has been accomplished by Walker [29] especially, and by Müller,[30] with both typhoid bacilli and cholera spirilla cultivated upon broth mixed with serum. Such strains not only increase in virulence but lose in both agglutinability and susceptibility to bactericidal effects. Sacquéppée [31] obtained similar results by keeping the organisms in collodium sacs in the peritoneal cavity, and Bail [32] found similar inagglutinability of typhoid bacilli taken from the peritoneal exudates of guinea pigs dead of infection.

Gay and Claypole in 1913 [33] produced the carrier state in rabbits with typhoid bacilli, and found that the organisms reisolated from the rabbits did not agglutinate in a serum which agglutinated stock

[29] Walker. *Jour. Path. and Bact.*, Vol. 8, 1902.
[30] Müller. *Münch. med. Woch.*, 1903.
[31] Sacquéppée. *Ann. Past.*, Vol. 4, 1901.
[32] Bail. *Archiv. f. Hyg.*, Vol. 42.
[33] Gay and Claypole. *Archiv. Intern. Med.*, 12, 1913, 614.

cultures in dilutions as high as 1:20,000. The writer, many years ago, noticed similar inagglutinability in a typhoid bacillus isolated from a case of gall stones which developed some 17 years after typhoid fever. A curious by-product of Gay and Claypole's observation is the fact that they claim that the blood and bile cultures which were not agglutinated by ordinary anti-serum were easily agglutinated by a serum produced by the immunization of rabbits with cultures grown on blood agar media. These observations, however, were not confirmed in an extensive investigation on this point by Bull and Pritchett.[34] The artificial production of inagglutinability by cultivation on immune sera was again extensively worked out in 1921 by Morishima [35] in our laboratory. Morishima's conclusions are as follows: Typhoid bacilli grown upon normal serum do not become inagglutinable. Cultivated continuously upon specific immune serum, they at first become inagglutinable, but if such cultivation is persisted in for two weeks or longer, eventually these strains again become agglutinable. In some cases this return to normal does not occur until the 72nd day; usually, however, it occurs sooner than this. The inagglutinability of the typhoid bacillus is accompanied by inability to absorb agglutinin. The development of capsular material does not seem to be concerned in the inagglutinability, since treatment by the Porges method, that is, washing with weak acid, does not render the inagglutinable strains agglutinable.

Zinsser and Dwyer [36] have noticed similar inagglutinability in typhoid bacilli recovered from the peritoneal cavities of guinea pigs injected with anaphylatoxin and bacteria. The anaphylatoxin in these cases possessed distinct aggressive action, and the conditions here were probably very similar to those observed by Bail.

There are various possible explanations, the most prevalent ones all representing variations of the opinion that such inagglutinable strains possess an inadequate receptor apparatus. Cole [37] advances this because he found these cultures possessed less power to absorb agglutinin than others, and, injected into animals, produced sera which were not highly agglutinating for the injected strain. Some of Cole's experiments show clearly the variable agglutinability displayed by different strains of the same species. Thus the agglutination in the same serum.

Of strain E = 1:8,000
Of strain H = 1:7,000
Of strain I = 1:4,500
Of strain W = 1:4,500
Of strain C = 1:4,000

[34] Bull and Pritchett. *Jour. Exper. Med.*, 24, 1916, 55.
[35] Morishima. *Jour. Bacter.*, 6, 1921, 275.
[36] Zinsser and Dwyer. *Proc. Soc. for Exp. Biol. and Med.*, Feb., 1914.
[37] Cole. *Zeitschr. f. Hyg.*, Vol. 46, 1904.

The difference here between E and C actually amounted to a relation of 1 to 2. A rabbit immunized with strain I furnished a serum which agglutinated strain E more powerfully than I itself.

Müller's experiments have the same general significance. It has also been suggested that the inagglutinable bacteria, especially those from the peritoneal exudate, which Bail found were neither agglutinable nor absorbed agglutinin, may have taken up altered agglutinin or agglutinoid. We will have occasion to recur to this problem in connection with our discussions of the capsulated bacteria and of virulence. The explanations given above do not seem on the whole satisfactory, and the problem is an exceedingly complex one. It has been found indeed that the acquired resistance of bacteria against agglutinins is not at all unique, and that acquired resistance against serum lysins may be observed.[38] The extensive investigations of Bail, Walker,[39] and others, on the nature of changes in virulence in many invasive bacteria, and the knowledge more recently gained on the resistance to phagocytosis of virulent strains of streptococci and pneumococci are facts closely related in underlying principle to the inagglutinability of typhoid strains cultivated in immune sera.

That no two strains of bacteria of the same species are exactly similar in their agglutinability in the same serum, moreover, is an observation which is made by every one who is in a position to carry out routine Widal tests in hospital practice. The spontaneous agglutination which occasionally occurs in the broth cultures of typhoid bacilli used for this test in many laboratories[40] may often be referred, at least in the cases which have come to the writer's notice, to an excessive acidity of the broth, a phenomenon which will be discussed in a subsequent paragraph. As far as the phenomenon of variable agglutinability inherent in different strains is concerned, however, it is of great practical importance in carrying out routine Widal tests to bear this in mind and to control the strain of typhoid bacilli employed in the reactions from this point of view. A strain also which has been in use for a long time should be titrated with agglutinating animal sera from time to time to determine whether or not alterations in agglutinability have occurred.

Group Agglutination.—That the reaction of bacterial agglutination was specific was noted, we have seen, by Gruber and Durham from the very beginning. The closer study of the reaction in its application to bacterial identification has led to interesting data which have revealed certain definite limitations of this specificity. It has been found, for instance, that, while immunization with any given species of bacteria gives rise to a very marked increase of agglutinins for this species, there are formed at the same time, though

[38] Eisenberg. *Centralbl. f. Bakt.*, Vol. 34, p. 739, 1903.
[39] Walker. *Centralbl. f. Bakt.*, Vol. 33, 1903.
[40] See section on Agressins.

to a lesser degree, agglutinins for bacteria of other species. This has been referred to as "group reaction," and the agglutinins appearing in such sera are spoken of by German observers as *"Haupt Agglutinine"* and *"Neben"* or *"Mit Agglutinine."* In English texts they are usually referred to as *"chief"* or *"major"* agglutinins and *"para"* or *"minor"* agglutinins. Although, as a general rule, such group-agglutinin formation is evident most markedly in the cases of biologically related micro-organisms like the typhoid, paratyphoid, and colon bacilli, this is not necessarily the case, and in some instances the immunization of an animal with a given bacterium may produce minor agglutinins for other bacteria that have no morphologically or culturally demonstrable biological relation to that which reacts with the major agglutinin. We may obtain the most graphic survey of these conditions by examining one of a number of experimental protocols in which such major and minor agglutinin formation is illustrated. Thus in the work of Hiss [41] on the dysentery bacilli the following relations were observed:

A serum produced in rabbits by immunization with the Shiga bacillus agglutinated the Shiga bacillus in dilutions of 1 to 20,000, the "Baltimore," "Harris," "Gray," and "Wollstein" bacillus 1 to 1,200, the Y bacillus and others 1 to 200.

An Anti-Y bacillus serum, which agglutinated this bacillus 1 to 6,400, agglutinated the Baltimore bacillus 1 to 1,600, and the Shiga bacillus 1 to 100.

Anti-"Baltimore" bacillus serum agglutinated this bacillus 1 to 3,200, and the Y bacillus 1 to 400, and the Shiga bacillus 1 to 100.

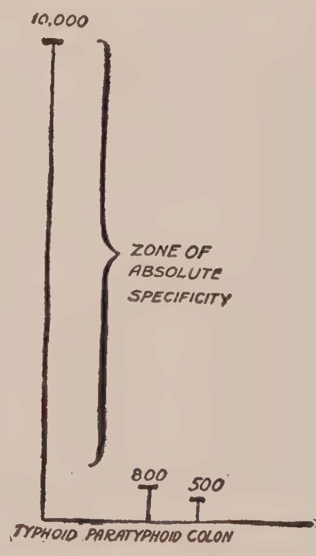
In this series there is fair correspondence between cultural biological relations and agglutination, and from many such investigations it would seem that "group" agglutination might be taken to represent a method of determining biological classifications similar to the zoölogical relations revealed by the precipitin reaction. While, in a general way, this is undoubtedly true, nevertheless great caution must be exercised in relying upon such evidence for classification, since notable exceptions have been observed. Park,[42] for instance, cites a case in which a horse, immunized with a paradysentery bacillus, agglutinated a colon strain in dilutions up to 1 to 10,000. Similarly Durham [43] found that two members of the colon group—one saccharose fermenting—reacted almost identically with the same agglutinating serum, while the agglutinations of two culturally identical bacilli of the hog cholera group were entirely at variance.

The cause for the phenomenon of group agglutination must unquestionably be sought in the nature of the bacterial agglutinogens, and it is but reasonable to assume that living cells so little differen-

[41] Hiss. *Jour. Med. Res.*, 13, N. S., Vol. 8, 1904.
[42] Park. "Pathogenic Micro-organisms," 1910, p. 166.
[43] Durham. *Jour. Med. Res.*, Vol. 5.

tiated biologically and morphologically should have much in common chemically as well. The bacterial cell, moreover, may contain several antigenic complexes and, beside its specifically peculiar constituents, therefore, we may suppose that every bacterium contains additional antigenic substances which it has in common with other species. It is the specific antigen in response to which the "chief" agglutinin is formed, while the others, present in smaller quantity, lead to the formation of the minor or para-agglutinins with an intensity proportionate to the amounts present in the bacterial cell. Thus, as Durham expresses it, if we assume one micro-organism to contain antigenic substances a, b, c, and d, and another d, e, f, and g, the antibodies produced by injections of the former would react with the common element d in the latter.

The diagnostic value of the specificity, however, is plainly not affected by the phenomenon of group agglutination, since the action of minor agglutinins can be always easily eliminated by sufficient dilution. Thus if we possess a typhoid-immune serum which agglutinates the typhoid bacillus in dilutions of 1 to 10,000, the paratyphoid bacillus 1 to 1,000 and the colon bacillus 1 to 100 (as in the figure), we may still utilize this serum for the identification of suspected typhoid cultures, as, let us say, in the isolation of unknown bacteria from stools or urine, by using potent sera in dilutions as high or higher than 1 to 1,000, beyond which point the action of minor agglutinins is eliminated. The diagram illustrates our meaning in the hypothetical case of a typhoid-immune serum which agglutinates typhoid in dilutions of 1 to 10,000, paratyphoid bacilli 1 to 800, and colon bacilli 1 to 100. The relation of agglutination to biologic relationship is not a simple problem in that individual strains even of the same species may vary considerably in agglutination by the same serum. Smith and Reagh [44] have studied particularly these conditions as they prevail in the colon, hog cholera and allied groups. They found that biologic relationship usually may be concluded from close agglutination affinities, and that minor biologic differences such as colony appearance, etc., do not



DIAGRAMMATIC REPRESENTATION OF GROUP AGGLUTINATION.

[44] Smith and Reagh. "Studies from the Rockefeller Institute," Vol. 1, 1904, p. 270.

exclude such affinities. On the other hand, closely related bacteria vegetating on mucous surfaces (different strains of diphtheria, dysentery, and colon bacilli) may vary considerably in their agglutinative characteristics, while invasive species show a greater homogeneity among their varieties or races. This brings in another important feature—that is, the modification in agglutinative characteristics induced in bacteria when they become parasitic upon different hosts, and Smith and Reagh conclude that such changes of parasitic habitat may modify the agglutinative properties (probably by adaptation to the peculiar reactions of each host), some of them being weakened and others strengthened.

The animal species used for immunization indeed influences the quantity and nature of the produced agglutinin considerably. For instance, in Pfeiffer's [45] experiments, a dog, a chicken, and a rabbit were immunized with the same strain of cholera spirilla. The sera obtained from these animals agglutinated this and other strains of cholera spirilla in an entirely irregular manner—showing that the constitution of the agglutinins in each case was an absolutely different one in regard to the relative concentrations of "major" and "minor" constituents.

Agglutinin Absorption.—Castellani [46] found that the immunization of an animal with two or more different species of bacteria results in the formation of agglutinins against all of these. Supposing, for instance, that species A and B are used for treatment, agglutinins against both A and B are formed in quantity, depending upon the intensity of the treatment in each case. Now, if to the serum so produced an emulsion of A is added, agglutinin A only will be removed, while agglutinin B will remain in the serum almost undiminished. An example of this is seen in the following protocol taken from Castellani's paper:

Titre of the serum	Titre after absorption with *B. typhi*	Titre after absorption with *B. coli* "31"	Titre after absorption with *B. coli* and *B. typhi*
B. typhi 4,000 *B. coli* "31" 1,000	*B. typhi* 0 *B. coli* "31" 1,000 > 300	*B. typhi* 4,000 *B. coli* "31" 0	*B. typhi* 0 *B. coli* "31" 0

In the preceding paragraphs, however, we have seen that immunization with a single organism, say *B. typhosus,* will induce the formation of agglutinins, not only for this species, but also of para or minor agglutinins for biologically similar strains as well. In such

[45] Pfeiffer. Quoted from Paltauf in "Kolle u. Wassermann Handbuch," Vol. 4.

[46] Castellani. *Zeitschr. f. Hyg.,* Vol. 40, 1902.

cases, as Castellani showed, absorption of the serum with the organism used for immunization takes out, not only the major agglutinins, but rather all of the agglutinins, major and minor. Conversely, however, absorption of such a serum with the species agglutinated by the minor agglutinins takes out these antibodies only, leaving the major substances intact. These relations are well illustrated by the two following protocols, also taken from Castellani's paper:

Serum of rabbit No. 1 immunized with *B. typhi* only	
Agglutination titre of serum	**Titre after absorption with *B. typhi***
B. typhi 5,000	*B. typhi* 0
B. coli 600	*B. coli* 0

Serum of rabbit No. 7 immunized with *B. typhi* only		
Agglutination titre of serum	**After absorption with *B. typhi***	**After absorption with *B. coli***
B. typhi 10,000 (heavy clumps)	*B. typhi* 0	*B. typhi* 10,000 (small clumps)
B. coli 800	*B. coli* 0	*B. coli* 0

Note: All of these protocols are taken from Castellani's communication, *loc. cit.*

These facts, variously confirmed, tend to corroborate the conception of the production of major and minor agglutinins outlined above.

It is of practical and theoretical importance to mention that complete absorption of specific agglutinin by a single exposure to homologous bacteria, however thickly emulsified, is not possible. It is always necessary to absorb repeatedly, and even then a minute trace of agglutinin may eventually remain. Eisenberg and Volk,[47] who have studied these conditions particularly, attribute this to the nature of the union of agglutinogen with agglutinin, which they conceive as following the laws of mass action—this accounting for the persistence of a small "rest" of free agglutinin, even after repeated absorption by partial dissociation. The principle involved here is identical with that discussed in connection with antigen-antibody union in general.

Normal Agglutinins.—It is not only in the sera of immunized animals, however, that agglutinins are found. Just as the other

[47] Eisenberg and Volk. *Zeitschr. f. Hyg.*, Vol. 40, 1902. Eisenberg. *Centralbl. f. Bakt.*, Vol. 34, 1903.

antibodies, antitoxins, and bactericidal sensitizers may be found in the blood of normal animals, so agglutinins for various bacteria may be normally present. These normal agglutinins do not in any respect, further than that of quantity, differ from the immune agglutinins and follow the same laws of specificity which have been described for the latter. It has been shown a number of times that such normal agglutinins are not present in the new-born animal, but are acquired later in life, possibly because of the absorption of bacterial products from the intestinal canal. It has been variously shown [48] too, that living bacteria themselves may enter the lymphatics and the portal circulation from the intestine during apparently perfect health of the individual.

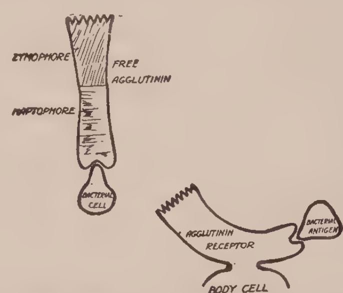
This subject is of interest, not only in connection with the agglutinins, but has bearing upon the existence of normal antibodies in general. Ruffer,[49] who has studied particularly the penetration of leukocytes and bacteria through the intestinal mucosa, demonstrated micro-organisms in the sub-mucous lymph nodes of normal rabbits, and Ribbert [50] and Bizzozero [51] have shown the presence of bacteria in apparently normal mesenteric lymph nodes. Adami and Nichols even claim that during health a certain number of living bacteria enter the portal circulation from the intestine, and from here may get into the systemic circulation, and are ordinarily destroyed by either leukocytes, liver lymphatic organs, or the kidneys.

It is thus not surprising that normal agglutinins should occur, and that they should be qualitatively identical with the so-called "immune" agglutinins, since they probably arise by a sort of spontaneous immunization through the intestinal canal. Researches of Ford [52] point in the same direction. However, Landsteiner and Reich [52a] in 1907 pointed out differences between normal and immune agglutinins in that the normal were more resistant and did not possess the specificity of the immune. For the details of these experiments, we refer to their publication. Their work prevents the unqualified acceptance of the identity of normal and immune antibodies.

Ehrlich's Conception of Agglutination.—An interpretation of the process of agglutination, according to the theory of Ehrlich, conceives it as a chemical union of agglutinin and bacteria (agglutinogen). The agglutinin is regarded as consisting of two atom complexes, one the "haptophore," having affinity for the bacterial protein, and concerned with the union, the other the "ergophore" or "zymo-

[48] Adami. *Jour. Am. Med. Assoc.*, Dec., 1899.
[49] Ruffer. *Brit. Med. Journal*, 2, 1890.
[50] Ribbert. *Deutsche med. Woch.*, 1885.
[51] Bizzozero. *Centralbl. f. d. Med. Wiss.*, Vol. 23, 1885, p. 49. Quoted from Adami.
[52] Ford. *Zeitschr. f. Hyg.*, Vol. 40, 1902.
[52a] Landsteiner and Reich. *Zeitschr. f. Hyg.*, Vol. 58, 1907, p. 213.

phore," by means of which the actual agglutination is brought about after the union has taken place. Unlike the antibody concerned in the processes of hemolysis or bacteriolysis, the agglutinins are not dependent in their action upon the coöperation of alexin, and the agglutination power of a serum is therefore not destroyed by inactivation or heating to 56° C., as is the case with the former. Although the accurate point of thermal destruction varies with different agglutinins (the agglutinins for the *Bacillus pestis* and a few other bacilli are said to be destroyed at 56° C.), as a rule agglutinins will not disappear from serum until the temperature is raised to between 70° and 80° C. Once destroyed, however, no reactivation takes place upon the addition of fresh normal serum. Ehrlich, for this reason, has conceived the structure of agglutinins as "*Haptines* of the Second Order," in which he supposes that the zymophore and the haptophore groups are inseparably connected, and in which we could assume an alteration of the less stable zymophore group without interference with the functional integrity of the haptophore group. Such an altered agglutinin could be spoken of as "agglutinoid," and this could become united with a bacterial cell without inducing agglutination, but, by its union, prevent subsequent combination of the cell with unaltered agglutinin. This process of "agglutinoid Verstopfung" has been held responsible for the failure of agglutination when bacteria that have been in contact with heated serum are subsequently exposed to the action of actively agglutinating serum. It is assumed that the agglutinoids which were present in the heated serum have occupied the bacterial receptors and have thereby prevented the union of these with the agglutinins later added.



Diagrammatic Representation of Ehrlich's Conception of the Structure of Agglutinin.

The So-called Agglutinoid Phenomenon.—The work of Eisenberg and Volk [53] has gone very thoroughly into these conditions and forms the strongest bulwark of this point of view. These workers showed that bacteria thus exposed have not only become less sensitive to agglutinins, but have, at the same time, lost much of their power to absorb agglutinins when compared with normal bacteria. The same loss of agglutinating power which is observable in heated agglutinating serum is evident to a lesser extent in serum which has been preserved at room temperature. Eisenberg and Volk have shown that such serum, in addition to a quantitative loss of agglutinin content, loses the power to agglutinate in high concentrations. Thus

[53] Eisenberg and Volk. *Zeitschr. f. Hyg.*, Vol. 40, 1902.

a serum which has been preserved in this way will no longer agglutinate bacteria in concentrations of 1 to 20, 1 to 40, or even 1 to 100, but will agglutinate as before in higher dilutions. This is taken to mean that the agglutinoids formed during the period of standing possess a higher affinity for the bacterial antigen than do the true unaltered agglutinins. Since these so-called "proagglutinoids" are relatively small in amount, their action is masked when considerable dilution has sufficiently diminished their quantity, in proportion to the more plentiful unaltered agglutinins. In support of this assumption it has been further shown that sera which have been rendered inhibitory by either of the methods named can be deprived of their inhibiting characteristics by absorption with homologous bacteria. Together these observations might appear to constitute a strong argument in favor of the agglutinoid theory.

In practical experience the existence of such an inhibition zone is of great importance, since freshly taken sera will occasionally show this failure of agglutination in concentration, while strong agglutination follows when the dilution is increased. In clinical tests, as in the Widal reaction for the diagnosis of typhoid fever, we not infrequently encounter sera which will give no agglutination in dilutions of 1 to 20 and even 1 to 40, and the reaction would therefore be regarded as negative unless the possibility of the proagglutinoid zone were recognized and further dilutions carried out.

While there is no question of the accuracy of the experimental data cited in the preceding paragraphs, the interpretation of the phenomena on the basis of Ehrlich's haptine conception has not been universally accepted.

The gist of the explanation of the so-called "agglutinoid" phenomena by the Ehrlich school, therefore, can be summarized as follows: The agglutinin is conceived as consisting of a "haptophore" and a "zymophore" group; the "haptophore" group brings about the specific union of the agglutinin with the bacteria, the "zymophore" group causes the agglutination. Heating destroys the "zymophore" group, but not the "haptophore" group. In consequence, such altered agglutinin or "agglutinoid" can unite with bacteria, but can no longer cause agglutination. By uniting with the bacteria, it occludes the bacterial receptors for agglutinin in such a way that normal agglutinin, added subsequently, is unable to unite with the bacteria at all. In the transformation of agglutinin to "agglutinoid," the affinity is changed so that the "agglutinoid" now unites more easily with the bacteria than does normal agglutinin, hence the term "proagglutinoid." There seems to us almost nothing in this theory which can justly be retained. In the first place, Bordet's salt experiment has pretty well excluded the assumption of "haptophore-zymophore" structure on the part of the agglutinin. Moreover, as we have seen in discussing complement fixation, and as we shall see in

discussing precipitins, the so-called "agglutinoid" zones are in entire analogy with the zone phenomena observed with other antibody reactions, and dependent upon principles of quantitative union between antigen and antibody that have nothing whatever to do with deterioration of the antibody by heat or otherwise. The only point of argument on the part of the Ehrlich school which is not easy to answer in this connection is the claim made by Eisenberg and Volk, as well as by Kraus and Joachim, that the agglutinin inhibiting properties of a heated serum can be specifically absorbed out of such a serum with the appropriate homologous bacteria. If the matter of heating were only active by producing a protective colloidal action in the serum, then one would have to assume that such heated serum would prevent agglutination of any bacteria, or that normal heated serum could do the same thing. If, however, as Kraus and Joachim and others have claimed, it is possible to produce agglutinin inhibiting properties in typhoid immune serum by heating and then specifically absorbing out this inhibiting substance with typhoid bacilli, in such a way that the serum is deprived of its "agglutinoid" action, by the bacilli, and the bacilli are rendered inagglutinable by virtue of the material they have absorbed out of the serum, it would appear as though the inhibiting substance were really some inherent alteration property of the specific agglutinin, itself. Our explanation of this is as follows: We know from experiments of Porges, as well as from our own, that in various colloidal precipitations in which serum is involved, moderate heating of the serum will strongly reduce its ability to precipitate a suspension. Thus, while minute amounts of fresh dog serum precipitate colloidal arsenic-sulphid, a very minute amount of heated dog serum will inhibit such precipitation by fresh dog serum. Now, when we heat any normal serum, it is likely that we are changing it in its colloidal state, and producing a certain amount of colloidal protective property in the serum. In all reactions by the bacteria and antiserum, it is likely that the antibody carries into the union a not inconsiderable amount of inactive serum constituents. Thus, in precipitin reactions, as we shall see, the bulk of the precipitate formed comes from the serum. It is our belief that the so-called specific action of "agglutinoids" is due to the fact that the antibody carries into the union with the bacteria inactive protein which has become colloidally protective by virtue of the heating.

Hemagglutination.—In describing the investigations which led to the discovery of the mechanism of the lytic phenomenon, in the chapter on Cytolysis, we mentioned that Bordet and others had noticed the frequent agglutination of red blood cells in the sera of animals treated with such cells after the hemolytic property had been destroyed by heating to 56° C. Such hemagglutination is a phenomenon entirely analogous to the agglutination of bacteria by

serum, and hemagglutinins regularly result when an animal is systematically treated with the red blood cells of another species. Like the bacterial agglutinins, the hemagglutinins are relatively thermostable and are best observed, therefore, after the sera are inactivated. Otherwise rapid hemolysis will often obscure the agglutination. Like other agglutinins the hemagglutinins thus produced are specific, acting only upon that variety of cells which are used in their production. Moreover, certain sera may normally contain hemagglutinins for the blood cells of animals of another species. An illustration of this is the hemolytic and hemagglutinating property of normal goat serum for rabbit cells—but there are many other examples which might be cited. Such normal hemolytic and hemagglutinating properties for the cells of other animals usually render the sera toxic for these animals, and some observers have attributed the toxicity to this agglutinating action, believing that the clumped erythrocytes form emboli around which clotting is initiated. The writer's own investigations, however, seem to show that this is not the case, since the toxicity of such sera is completely removed after they have been heated, in spite of the fact that the hemagglutinative property remains unchanged.

Bordet's Analysis and the Importance of Electrolytes.—The fundamental principle underlying all the Ehrlich hypotheses is the conception that these reactions take place as do purely chemical reactions, following the law of multiple proportions. Such reasoning has often necessitated the assumption of differences of affinity which, critically examined, are really *ex post facto* explanations, forcing the phenomena to conform with the theory. As a matter of fact, the bodies which participate in the antibody-antigen reactions are probably of the nature of the substances which are spoken of as colloids, and it is therefore more than likely that the quantitative and other relations governing the union of these reagents should be analogous to those governing colloidal reactions in general. The reaction of agglutination, like that of precipitation, has lent itself particularly to the study of the principles of the union from this point of view, and the first and fundamental progress made in this direction is found in the work of Bordet.

Bordet [54] compared the formation of precipitates in bacterial emulsions to the precipitation of such inorganic emulsions as clay in distilled water, and noted that the precipitation of homogeneous emulsions of such substances is "often controlled by such insignificant causes as the presence of salts." Applying this analogy to the agglutination of bacteria, he performed the following experiment: Cholera spirilla, emulsified in salt solution, were treated with homologous immune serum and, after agglutination had taken place, the bacteria were thrown to the bottom by centrifugation and divided

[54] Bordet. *Ann. de l'Inst. Pasteur,* 1896, 1899.

into two parts. One part was again suspended in salt solution, and the other was washed, and then suspended in distilled water. The bacteria in the tube of salt solution rapidly agglutinated, while those in the distilled water, after thorough shaking, remained indefinitely suspended in an even emulsion. If, however, to these unagglutinated bacteria a small amount of pure sodium chlorid was added agglutination occurred.

The conclusions that can justly be drawn from this experiment are, first, that the bacteria could not agglutinate, even though they had been bound to agglutinin, when salt was removed from the environment, and, second, that the addition of salt to such emulsions brought about immediate agglutination. The same principle can be demonstrated in other ways. If, for instance, a bacterial emulsion is rendered free of salt by dialysis, and this is added to an agglutinating serum similarly dialyzed, no agglutination occurs. The suspension may remain evenly clouded indefinitely unless salt is added. As soon as a little salt is added, however, perfect agglutination occurs. To this technique the very obvious criticism may be applied that perhaps the absence of salt has precipitated the agglutinins, which, as we know, are precipitated with globulin, which is insoluble in the absence of salt. However, this source of error is excluded by the first experiment cited, and, moreover, it can be shown by the last experiment that, even though the bacteria are not agglutinated in the salt-free serum, they have nevertheless absorbed agglutinin. For, if such a salt-free mixture is centrifugalized, the bacteria washed and suspended in distilled water, and salt is then added, agglutination occurs. The supernatant fluid of the original suspension, furthermore, can be shown to have been deprived of agglutinins by suitable experiment.

These facts, first observed by Bordet, and further elaborated by the studies of Joos,[55] Friedberger,[56] and others, have been interpreted in different ways. Joos claims that there is a chemical union between the bacteria and the salt, and bases this upon the observation that the salt added to a salt-free mixture cannot be demonstrated in the supernatant fluid after agglutination has taken place. His observations in this respect have not found confirmation at the hands of Friedberger and other workers, and it is generally agreed to-day that the rôle of the salts is, as Bordet first assumed it to be, a purely physical one. Bordet's opinion is often spoken of as the *"two phase"* theory, in that he conceives the process of agglutination to consist of two distinct occurrences, first, an absorption of the agglutinin by the bacteria, and, second, an agglutination of the new complex by the salt. It is not the agglutinin which causes agglutination, but by union with the agglutinogen forms a complex which is altered in

[55] Joos. *Centralbl. f. Bakt.*, 1, Vol. 33, 1903.
[56] Friedberger. *Berl. klin. Woch.*, 1902; *Centralbl. f. Bakt.*, 1, Vol. 30.

"colloidal stability," and therefore is flocculable by the action of the electrolyte.

The opinion of Bordet becomes clearer as we consider the conditions governing the flocculation of colloids in general. Without wishing to enter in this place into detail regarding the nature of colloidal suspensions, it nevertheless seems necessary in order to do justice to this phase of the question to recall briefly the conditions governing such flocculation. The so-called colloidal solutions are not true solutions as the term is applied to dissociable substances, but are looked upon as consisting of small particles in suspension. The particles are similarly charged, as can be demonstrated by their wandering when subjected to an electric current, and it is supposed that it is this fact of similarity of charge which, in the "sol" state, permits them to remain in suspension. For the similarity of the charges of the individual particles prevents their mutual approximation.[57]

The state of suspension of these substances, then, represents a delicately balanced equilibrium between the two forces of electrical repulsion and of surface tension, an equilibrium which may be disturbed by the action of a number of factors. Thus, studies on inorganic colloids have shown, long before these considerations were applied to the explanation of serum reactions, that the stability of these suspensions could be disturbed both by electrolytes and by the addition of other colloids. Thus they may be precipitated by various salts, acids, and bases and, as Schultze[58] has shown, they react with that ion of the electrolyte which carries an opposite charge to that of the colloidal particles. For, although the colloidal units are similarly charged, this may be either negative or positive, according to the nature of the particular substance. In the case of the so-called amphoteric colloids reaction may take place, according to Pauli,[59] with both ions of the electrolyte.

The probable mechanism of the process is postulated by Pauli in describing the precipitation of a colloidal metal by salts, acids, or bases in the following way:

"The introduction of such electrolytes into a colloidal suspension is of course accompanied by a certain amount of dissociation. In consequence the weakly charged particles of the colloid collect about the ions of opposite charge until a sufficient accumulation of the particles leads to an electrical neutralization of the ion, and the

[57] That these relatively simple conceptions of the conditions in suspensions containing proteins and other colloids call for considerable revision in view of Loeb's recent work will be discussed in a later section. They are crudely stated here because much of the work on agglutination cited below was based upon them.

[58] Schultze. *Jour. f. prakt. Chem.*, 25, 1882, and 27, 1883.

[59] Pauli. "Hofmeister's Beiträge," 1905, and "Physical Chemistry in Medicine," Wiley & Son, N. Y., 1907.

aggregation, if of sufficient size, will sink to the bottom, forming a precipitate."

In regard to the mutual influences exerted upon each other when two colloids are mixed, it has been shown by Biltz, Hardy, and many other observers that oppositely charged colloids precipitate each other, though this is not an absolute rule, as experiments by Professor Stewart Young, of Stanford, have shown. Thus colloidal gold and platinum will be precipitated by such colloids as ferric oxid or aluminum oxid. When such a precipitating colloid is added tc another oppositely charged suspension in quantities too small to bring about flocculation, moreover, the addition of a quantity of salt, likewise too small to precipitate alone, will in many cases bring about the flocculation.

These and other phenomena of colloidal reaction have found close analogy in antibody-antigen studies, and have given support to the interpretation of the latter in the sense of Bordet.

To return to the consideration of bacterial agglutination, we have spoken of the dependence of the reaction upon the presence of salts, and have seen that the researches of Friedberger and others have refuted the assumption that the action of the salt in bringing about agglutination depends upon chemical union of the salt with the bacteria. It is probable, therefore, that here, as in other colloidal precipitations, the function of the salt is to be regarded purely as an electrophysical phenomenon.

The analogy becomes still closer when we consider the researches of Bechold,[60] Neisser and Friedemann,[61] Sears and Jameson,[62] and others, which have shown that bacteria in suspension are to be compared very closely with true colloidal suspensions in that the bacterial cells carry a definite and uniform electrical charge and wander in the electric field.

Bacteria in salt solution emulsion, for instance, wander to the anode, thus giving evidence of their carrying a negative charge. This charge may be altered by adding to the emulsions definite concentrations of acids or bases, a reversal of the charge taking place under the influence of NaOH or other hydroxids. Just how this is brought about is by no means clear, but it is not impossible that there is a selective absorption of OH ions by the bacteria, which therefore take on the charge of the ion.

However this may be, and we must admit that explanations of these phenomena are as yet largely speculative, a fact which interests us particularly in connection with the phenomena under discussion at present is the influence exerted upon the charge of bacteria by

[60] Bechold. *Zeitschr. f. physik. Chem.*, 48, 1904.
[61] Neisser and Friedemann. *Münch. med. Woch.*, Vol. 51, pp. 465, 827, 1904.
[62] Sears and Jameson. "Thesis for M. A., Stanford University," 1912.

exposure to the influence of serum. Bechold,[63] as well as Neisser and Friedemann,[64] assert that bacteria which have absorbed agglutinin no longer wander to the anode, but act as though they had been deprived of electrical charge, and precipitate or agglutinate between the electrodes.

Bechold has suggested, for this reason, that it may be possible that bacteria in the normal condition are protected from the action of the electrolyte by a membrane or coating of protoplasm which acts as a protective colloid. The absorption of agglutinin may alter this in such a way that they become amenable to the flocculating effects of the salt ions. In keeping with such an opinion is the well-known observation of the inagglutinability of capsulated organisms, which, as Porges[65] has shown, become agglutinable as soon as the capsules have been destroyed by heating in a weak acid.

That the absorption of agglutinin indeed alters the electric stability of the emulsified bacteria further appears from the fact that "agglutinin" bacteria[66] are agglutinated by concentrations of salts which are too slight to affect the normal micro-organisms. In this respect there is close similarity between the flocculation of agglutinin-bacteria and such emulsions as kaolin and mastic, whereas bacteria without agglutinin require much higher concentrations of the salts to produce like effects. The absorption of agglutinin may have removed a factor which protected the bacteria against the influence of the salt. On the other hand, it is equally just to assume—and this is more likely and corresponds with Bordet's views—that the absorption of agglutinin has "sensitized" the bacteria to the action of the electrolyte. The experimental facts upon which the above statements are formulated are largely found in the important papers of Neisser and Friedemann—whose work brought out, likewise, interesting analogies of the colloidal precipitations with the phenomenon which we have described above as the proagglutinoid zone.[67]

In regard to the greater amenability of agglutinin bacteria to flocculation by electrolytes, the following protocol, adapted from the work of these authors, will explain itself. They were tabulated from experiments in which different quantities of normal $\frac{n}{1}$ solution of various salts were added, on the one hand to emulsions of unaltered bacteria, and, on the other, to bacteria which had absorbed agglutinin. It is seen that, with some salts, agglutination of the unaltered bacteria did not occur at all, whereas agglutination was

[63] Bechold. "Die Kolloide in der Biologie u. Medizin," Steinkopf, Dresden, 1912.

[64] Neisser and Friedemann. *Münch. med. Woch.*, Vol. 51, 1904, pp. 465 and 827.

[65] Porges. *Zeitschr. f. exp. Path. u. Therapie,* 1905.

[66] "Agglutinin" bacteria—bacteria which have absorbed specific agglutinin.

[67] See work of Northrop and that of Coulter in its bearing on this point in subsequent sections on pages 267 and 313.

brought about in the treated bacteria with comparatively small amounts; in other cases the difference is a quantitative one only:

Protocol constructed from the tables of Neisser and Friedemann, loc. cit.

$\frac{n}{1}$ solution of salt	Quantity of salt sol. which brought about agglutination of 1 c. c. of *normal bacteria* in emulsion. 0 = no agglutination by the salt solution	Quantity of salt sol. which agglutinated 1 c. c. of *agglutinin bacteria* in emulsion
$NaCl$	0	.025
$NaNO_3$	0	.025
Na_2SO_4	0	.025
RbI	0	.025
$MgSO_4$	0	.0025
$ZnSO_4$	.01	.001
$CaCl_2$	0	.005
$BaCl_2$	0	.005
$Cd(NO_3)_2$	.01	.001
$CuSO_4$	.0025	.0001
$CuCl_2$	.0025	.0005
$Pb(NO_3)_2$	.0025	.0001
$HgCl_2$	.0025	.0005

The analogy between the experiment tabulated in the preceding protocol and the following from the work of the same writers is self-evident. Just as the absorption of agglutinin by bacteria rendered these more amenable to precipitation by salts, so the addition of minute quantities of gelatin to mastic emulsions had a similar sensitizing effect upon these.

NaCl 10% solution	1 c. c. mastic (1–10 original emulsion) diluted to 3 c. c.	1 c. c. mastic + .0001 c. c. of a 2% gel. solution, the whole diluted to 3 c. c.
1.0	+++	+++
0.5	0	+++
0.25	0	+++
0.125	0	+++
0.05	0	0
0.025	0	0

Finally, one of the most important analogies yielded by the work of the above investigators is illustrated in the following protocol as follows:

Colloidal iron hydroxid	Precipitation of mastic emulsion 1 c. c.
1.	0
0.5	0
0.25	0
0.1	++
0.05	+++
0.025	+++
0.01	+++
0.005	+++
0.0025	++
0.001	0

Here we have an inhibition zone in the tubes containing the highest concentrations, accurately analogous to the previously discussed *proagglutinoid* zone. It is a phenomenon similar also to the inhibition zones noticed in precipitin reactions and observed, though by a different technique, in bacteriolytic phenomena discussed in another place in connection with the Neisser-Wechsberg notion of complement-deviation or "Komplement Ablenkung." It seems to be a universal fact governing the union of colloidal substances, that definite quantitative proportions must be maintained in order to lead to reaction, this being, possibly, explicable on the basis that actual union can take place only after disturbance of the electrical balance which keeps the particles apart. These reactions will be found more accurately discussed in another place. Whatever the mechanisms may be, however, these and similar experiments have seemed to render unnecessary and unlikely the assumption of proagglutinoids, proprecipitoids, etc., to explain the inhibition zones so frequently observed in all reactions of this kind.

A peculiar observation, which does not coincide with the above interpretation of these phenomena, and the significance of which is indeed doubtful, is one which Friedberger [68] made in researches in which he confirmed the work of Bordet on the absence of agglutination in a salt-free environment. He found that not only the addition of various salts would bring about agglutination under such conditions, but that organic substances such as dextrose and asparagin could be substituted for salts and had similar agglutinating effect—although higher concentrations of these than of the salts were required. Were these substances at all dissociable it might be possible that they acted by a mechanism identical with that of the salts—but since such substances as dextrose either do not dissociate at all or do so to an infinitesimal degree only there does not seem any possibility of reconciling these results with Bordet's theory.

[68] Friedberger. *Centralbl. f. Bakt.*, 30, 1901.

It is difficult to explain Friedberger's results. Possible impurity of his preparations and the presence of traces of electrolyte seem to be excluded by the fact that he was quite conscious of this possibility of error and used only substances which yielded no ash on combustion.

It may be that the results of Friedberger in which glucose and asparagin were used may have brought about agglutination by an entirely different mechanism from that which we are discussing and form no analogy to this.

The most important recent piece of work that has been done on agglutination in our opinion is that of Northrop and deKruif.[69] As we have seen above, it is well known that bacteria, as well as other particles in suspension, carry an electric charge with reference to the surrounding medium, and we have already referred to the idea that repellent forces due to the like charges carried by individual particles, represented the force by which agglomeration is prevented. As we have mentioned above, and as will be seen further discussed in the chapter on colloids, the two forces which are opposed to each other in suspensions of this nature are the electric charge above alluded to, on the one hand, and the surface tension which has been supposed to exert a force of attraction between particles. It is the balance between these two which until very recently was regarded as determining suspension or precipitation. The question of the charge on suspended particles has been investigated by a number of workers, a particularly interesting paper being that of Powis,[70] who studied oil emulsions and found that coagulation occurred when the potential between the drops of oil and the surrounding liquid was reduced below a critical value of about 30 millivolts. Thus, the stability of the oil emulsion was definitely shown to depend upon the potential. Northrop mentions, however, that in the case of bacteria, this cannot be taken to have a direct bearing because agglutination by immune serum can occur without any apparent change of potential. For the first time in the investigation of agglutination, at least as far as we know, Northrop and deKruif have taken into account, in addition to the charge on the bacteria, the cohesive forces, and have attempted to measure both of these factors in relation to each other.

They worked particularly with thoroughly washed suspensions of B. typhosus and the Bacillus of rabbit septicaemia. The potentials were calculated from the rate of migration of the organisms in an electric field.

The cohesive force they measured by the following interesting technique which we quote verbatim.

"*Measurement of the Cohesive Force.*—A piece of thick glass slide was covered with a thin film of very heavy suspension of washed

[69] Northrop and deKruif. *Jour. Gen. Phys.*, 4, 1922, 639.
[70] Powis. *Zeit. f. Physik. Chem.*, 89, 1914, 186.

organisms (B. typhosus), the film allowed to dry and then heated to 60° for a few minutes. This causes the bacteria to adhere firmly to the glass. A heavy (No. 3) cover-slip was similarly prepared. The cover-slip was suspended by means of a fine platinum wire from the lever of the de Noüy [71] surface tension apparatus. The glass slide was immersed in a dish containing the solution to be studied and the cover-slip allowed to rest on it with its own weight for 1 minute. The force required to pull the cover-slip from the slide was then determined. It was found that if the measurement was made immediately after the two surfaces came in contact, the value obtained depended on the force with which the two had been pressed together. If the slip had been pressed down firmly a much greater force was required than if it had simply been allowed to rest on the slide. After a short time interval, however, this difference became less, and eventually the same reading was obtained in both cases. This is due presumably to the fact that the distance apart of the two surfaces is regulated by capillary forces and comes to the same point from either side. The same smear was used as long as the same value was obtained on replacing the preparation in distilled water. The value obtained becomes less after ten or fifteen measurements, due to the gradual removal of the film. Control experiments with clean glass surfaces showed no significant variation under the conditions of the experiment. The values obtained in this way were surprisingly reproducible. They have been expressed as milligrams required to separate two surfaces each 2 cm. square. The results are not exactly comparable to the measurements of the potential since the organisms have been subjected to dry heat. It will be noted, in fact, that the results do not conform exactly to those expected from the potential measurements. In the case of NaCl, for instance, the concentration required to affect the cohesive force noticeably, is slightly higher than would be expected from the potential curve.

"It has usually been considered that this force is a surface tension effect, but there does not appear to be any conclusive evidence as to its nature. It is better, perhaps, to refer to it simply as 'cohesive' without an exact definition of its nature."

The conclusions they reached in their work are extremely interesting and considerably at variance with many of the ideas previously held. In examining the effects of salts on the agglutination of washed bacteria, it was found that their experiments could be divided into two groups: first, those in which agglutination was caused by very low concentrations, less than 0.01 normal, and those in which high concentrations were needed. In the first group, it was found that agglutination occurred whenever the potential between the organisms and the solution was reduced below 13 millivolts, either positive or negative. This indicated that the change of potential

[71] deNoüy. *Jour. Gen. Physiol.*, 1, 1918, 521.

was the essential factor. In the second group, however, agglutination with many salts did not occur, even though the potential was reduced to an immeasurably small value, and in such cases they assume that the salt affected the cohesive force as well as the potential. This supposition was confirmed by experiment. In other words, they found that high concentrations of salts decreased the cohesive force. Therefore, although the potential required to keep them apart was also decreased and in concentrated salt was reduced to zero, the cohesive force was rendered so insignificant that agglutination did not occur.

In regard to serum, they showed that if dialyzed serum is added to a suspension of organisms in distilled water, the potential of the organisms gradually decreases, but not enough to cause agglutination. If a salt solution now is added to this mixture until the potential is decreased to the critical value of 13 millivolts, agglutination occurs. The serum seems to prevent the salt from decreasing the cohesive force between the organisms, and the potential, therefore, determines the agglutination when the salt becomes sufficiently concentrated to reduce this to less than 13 millivolts.

Conversely, if the experiment is reversed, that is, if serum is added to organisms in the presence of salt, sufficient in amount to show this low potential, the serum increases the cohesive force until it is greater than the repulsion due to the potential (which latter is not affected by the addition of serum), and agglutination occurs.

Of course, their work does not touch upon the possible reasons why immune sera alter the bacteria more definitely than normal sera, or upon what the specific absorption of immune bodies from serum depends, but their work brings us closer to an understanding of the physical conditions which underlie the agglutination of bacterial suspensions.

Acid Agglutination.—In one of the preceding paragraphs we have mentioned the phenomenon spoken of as "acid agglutination." By this is meant the spontaneous clumping, not only of bacteria, but of small particles of any kind, in suspension, in the presence of certain concentrations of acid. Michaelis,[72] Beniasch,[73] and others who have studied this phenomenon in detail have come to the conclusion that it is the concentration of the hydrogen ions which is responsible for the agglutination. This explanation is also applicable to the agglutination often observed about the anode when bacteria are subjected in suspension to the action of a direct current. In such experiments the organisms after concentrating at this electrode often flocculate, and it is here, of course, that hydrogen ions are present in the greatest concentration. How this takes place is problematical, but the reasoning of Pauli, if applied to this, would favor

[72] Michaelis. *Folia Serol.*, 7, p. 1010, and *Deutsche med. Woch.*, 37, 969.
[73] Beniasch. *Zeitschr. f. Imm.*, Vol. 12, 1912.

the assumption that the weakly charged bacteria group themselves about the ions and, when a sufficiently large aggregation has formed, fall to the bottom as precipitate. This phenomenon of acid agglutination is of course entirely different in nature from the specific serum agglutination which we are discussing. Nevertheless, Schidorsky and Reim,[74] Jaffe,[75] and others have attempted to apply acid agglutination to the isolation and differentiation of bacteria, on the conception that different species are agglutinated by varying concentrations of hydrogen ions. The former investigators, even, claim to have been successful in isolating typhoid bacilli from the stools by this method in that the typhoid bacillus was agglutinated by concentrations of acid which had no effect upon the *Bacillus coli.* Sears [76] has gone over this work carefully, and, while he has obtained results which bear out the contention that the agglutination is probably due to the concentration of the H ions, his experiments have revealed an irregularity in the behavior of bacteria of the same species in acid solutions and an overlapping of those of one species with those of another. Therefore the use of acid agglutination for differential purposes seems to us entirely hopeless. And indeed it would be surprising if any such distinctive and regular reaction differences between simple bacterial cells, after all chemically and physically so essentially alike, could be found.

The Functional Importance of Agglutination in Infectious Disease.—Since the property of specifically agglutinating bacteria is developed in the bodies of animals and man in the course of contact with bacteria in the course of infection, it is natural to speculate concerning the possible significance of this phenomenon. Of course, it is quite possible that it is merely an incidental, inconsequential effect of reactions between antigen and antibody, and it has been so looked upon by a number of investigators, such, for instance, as Metchnikoff,[77] who says the part played by agglutination in acquired immunity is "merely accidental and subordinate." Salimbeni [78] goes so far as to state that bacterial agglutination does not take place within the animal body. His work was done with cholera spirilla. On the other hand, many observers have actually determined agglutination *in vivo,* the first observation of this kind emanating from Sawtschenko and Melkich,[79] who found clumps of the spirochætes of relapsing fever in the blood of infected patients. Also, in natural immunity, such as that, for instance, of pigeons against pneumococci, Keyes and others have seen clumps of bacteria in the capillaries of

[74] Schidorsky and Reim. *Deutsche med. Woch.,* Vol. 38, p. 1125.
[75] Jaffe. *Arch. f. Hyg.,* Vol. 76.
[76] Sears. *Proc. Soc. of Exp. Biol. and Med.,* 1913.
[77] Metchnikoff. "Immunity in Infectious Disease," Cambridge, 1905, p. 263.
[78] Salimbeni. *Ann. de l'Inst. Past.,* 11, 1897, 277.
[79] Sawtschenko and Melkich. *Ann. de l'Inst. Past.* 15, 1901, 497.

the liver and spleen. Recently, Bull [80] has studied the matter in more detail. Bull finds that with typhoid bacilli, in the circulating blood of normal rabbits, they are rapidly clumped, removed from the blood stream and accumulated in the organs where they are taken up by polymorphonuclear leukocytes, in the liver and spleen, especially. In actively immunized animals, this is still more noticeable, both for the organisms mentioned, as well as for pneumococci, staphylococci, Shiga dysentery bacilli, and some other organisms. Bull's observation in regard to the action of pneumococcus immune serum, in pneumococcus infected rabbits, is particularly interesting in that he found that the injection of sufficient amounts of anti-pneumococcus serum into septicæmic rabbits resulted in a rapid clumping of the pneumococci in the blood stream. Incidental to other experiments, we have had occasion often to confirm this observation, and have been astonished by the speed with which the phenomenon occurs. Thus, if one injects a rabbit with pneumococci, allows him to go until the organisms are plentiful in the circulation, and then injects intravenously a sufficient dose of anti-pneumococcus serum, an immediate heart puncture will reveal clumps of the bacteria which were evenly distributed in the smears taken just before the injection. If one waits for 3 or 5 minutes after the serum injection, it may not be possible to find any organisms in the circulating blood by ordinary smear method.

It will appear from this work that the clumping of bacteria in the body of the infected animal may have a very definite protective function, consisting in a preliminary concentration of the bacteria in the capillaries of various organs where phagocytosis is, in consequence, facilitated.

[80] Bull. *Jour. Exper. Med.*. 22, 1915, 475 and 484; also, 24, 1916, 25.

CHAPTER X

THE PHENOMENON OF PRECIPITATION
(Precipitins)

The establishment of the agglutinin reaction as a constant and specific serum-phenomenon by the work of Gruber and Durham led immediately to assiduous investigation of the many problems suggested by it, and among them, as we have seen, the question of the nature of the agglutinogen. It was found that agglutinins could be produced, not only by the injection of whole bacteria, but equally as well by treatment with dissolved bacterial extracts or with filtrates from old broth cultures. This naturally led to the thought that there might be a definite reaction if such extracts (instead of the bacteria themselves) were added to agglutinating sera *in vitro*. Rudolf Kraus [1] was the first to perform this very logical experiment. He was working with broth filtrates of *Bacillus pestis* and of the cholera spirillum, and found that when he mixed the perfectly clear filtrates of such cultures with their respective antisera the mixtures would at first become turbid and finally show a light flocculent precipitate. He named the reaction the "precipitin reaction" and, in analogy to agglutinins, spoke of the bodies in the serum which caused the precipitation as "precipitins." The reaction was found, like that of agglutination, to be specific; the cholera serum gave no precipitate with the plague extract and *vice versa,* and Kraus, after extending his observations to other bacteria, pointed out the practical diagnostic possibilities of his discovery.

Though Kraus' first observations were made entirely with bacterial culture filtrates and antibacterial sera, it was soon discovered that his results were merely isolated instances of a broad biological law, and that specific precipitins were produced whenever animals were treated with injections of any kind of foreign protein. Thus Tschistovitch,[2] in 1899, found that the blood serum of rabbits immunized with eel-serum gave specific precipitates when mixed with eel-serum, and Bordet [3] obtained analogous results by treating rabbits with defibrinated chicken blood and with milk. Thus rapidly the discovery of Kraus was developed into the generalization that

[1] R. Kraus. *Wien. klin. Woch.,* No. 32, 1897.
[2] Tschistovitch. *Ann. de l'Inst. Past.,* 13, 1899.
[3] Bordet. *Ann. de l'Inst. Past.,* Vol. 13, 1899, pp. 225-273.

the sera of animals that have been treated with foreign proteins of any kind—bacterial, animal, or vegetable—will develop the property of causing precipitates when mixed with clear solutions of the respective antigens.

The substances which, after injection into the animal body, lead to the formation of precipitating antibodies are spoken of in the language of immunology as "precipitinogen." In the case of bacteria it has been shown that, while the injection of the whole bacterial cell—dead or alive—will lead to precipitin formation, bacterial extracts produced in a variety of ways will lead to the same result. Such precipitinogen extracts can be obtained by allowing the bacteria to grow in flasks of slightly alkaline bouillon, keeping them in the incubator for from three weeks to three months, and then filtering them through Berkefeldt candles. Again, useful extracts can be more rapidly produced by growing large quantities of bacilli on agar, emulsifying in salt solution, and shaking in any one of the ordinary types of shaking machine for 48 hours or longer. On filtering an extract is obtained which will form precipitates with homologous immune serum, or will incite precipitins when injected into animals. In fact, any one of the customary vigorous methods of extracting bacterial or other cells will yield precipitinogen. A relatively purified precipitinogen in the form of a dry, water-soluble powder has been obtained by Pick by the precipitation of culture filtrates with alcohol.

Precipitinogens.—Regarding the chemical nature of the precipitin-inducing substances, or precipitinogens, the same problems have arisen which have been discussed in connection with antigens in general. We may say that all soluble native proteins possess precipitin-inducing properties. Yet this does not sufficiently define the term, since many observations have been published which show that physically and chemically altered proteins may still induce specific precipitins; a few investigators, furthermore, have claimed that they have produced non-protein precipitinogen by various methods of breaking up the molecule of the original antigen. In the section on agglutination we have seen that moderate heating (56 to 65° C.) rather increases than decreases the agglutinogen characteristics of bacteria, and it is equally true that such heated bacteria or bacterial extracts may induce precipitins. The effects of various degrees of heat upon the specificity of precipitinogens have been extensively investigated and will be discussed, in considerable detail, below.

Of more immediate, indeed of fundamental, importance is the problem of a non-protein antigen. The most important claims in this regard have been made by Pick,[4] Obermeyer and Pick,[5] and by

[4] Pick. "Hofmeister's Beiträge," Vol. 1, 1901.
[5] Obermeyer and Pick. *Wien. klin. Woch.*, 1904, p. 265.

Jacoby.[6] Jacoby, working with a vegetable antigen, ricin, found that by trypsin digestion he could obtain a substance which still retained antigenic properties, but no longer gave any of the protein reactions. Obermeyer and Pick, by the same method, claim that they have produced a non-protein precipitinogen from egg albumen. On the other hand, others have had negative results, and Kraus [7] himself, after reviewing the evidence on both sides, comes to the conclusion that available data do not justify us in separating the antigenic properties from the protein molecule. In unpublished experiments which the writer carried on in the laboratory of Professor Friedemann in Berlin also attempts to produce a non-protein precipitinogen from horse serum by bacterial putrefaction were entirely negative. The putrefaction of the serum, though carried out in dialyzing bags for the removal of diffusible products, was extremely slow, and when finally the Biuret reaction disappeared the serum was no longer precipitable by potent antisera. However, the flaw in these experiments is that the true test of the presence of precipitinogen is not the precipitable character of the solution in question, since actual precipitation is dependent, as we shall see, upon many modifying secondary factors, but rather the ability of the substance to induce precipitins in treated animals.

The fact that Nicolle,[8] and later Pick,[9] were unable to obtain alcohol-soluble substances from bacteria and bacterial extracts which were still precipitable might also be taken to point toward the non-protein character of the precipitinogens, suggesting that these substances may be of a lipoidal nature. However, as Landsteiner [10] points out, mere solubility in organic solvents can no longer be taken as a proof of lipoidal character, since it is more than probable that non-lipoidal substances may go into alcoholic and other organic solution when lipoids, such as lecithin, are present. Thus Müller [11] found that the antigen of typhoid bacilli was soluble in chloroform in the presence of old preparations of lecithin. Pick and Schwartz,[12] who had previously studied similar antigen solubilities in the presence both of lecithin and of other organ lipoids, suggest that possibly such solutions represent lipoid-protein combinations—colloidal "solutions"—which permit the presence of protein mechanically or chemically united to the lipoid in the organic solvents—alcohol, chloroform, etc. Here, too, then there is no evidence for the existence of non-protein precipitinogen.

[6] Jacoby. "Hofmeister's Beiträge," Vol. 1, 1901. Oppenheimer. "Hofmeister's Beiträge," Vol. 4, 1904, p. 259.

[7] Kraus in "Kolle u. Wassermann Handbuch," Vol. 4, p. 605.

[8] Nicolle. *Ann. de l'Inst. Past.*, 12, 1898.

[9] Pick. "Hofmeister's Beiträge," Vol. 1, 1901.

[10] Landsteiner. "Weichhardt's Jahresbericht," Vol. 6, 1910, p. 214.

[11] Müller. *Zeitschr. f. Imm.*, Vol. 5, 1910.

[12] Pick and Schwartz. *Biochem. Zeitschr.*, Vol. 15, 1909.

In regard to the actual precipitable materials in the bacterial bodies, our own opinion is that they are not represented in the ordinary coagulable proteins alone. We do not wish to repeat too extensively observations reported in another part of this book. We, therefore, refer the reader to the section on alexin fixation, where we have set forth the manner of producing certain alcohol precipitable bacterial extracts from which all the ordinary proteins had been removed by boiling with acid. The substances there described, the chemical analysis of which is now occupying our own attention, are specifically and very sharply precipitable by homologous antiserum. Indeed, in the case of such organism as pneumococci, meningococci and influenza bacilli, the reactions are sharper and more powerful than with whole bacterial extracts in which adventitious substances seem to exert some inhibiting influence.

Of importance in connection with the problem of the nature of precipitinogen, also, is the claim of Myers,[13] that specific precipitins may be produced in rabbits by treatment with Witte peptone, a substance complex in constitution, but consisting largely of albumoses. This observation has failed of confirmation in the hands of Obermeyer and Pick, Michaelis,[14] Norris,[15] and others, and cannot, therefore, be accepted as an established fact.

Whichever method of precipitinogen production is used bacterial precipitins appear in the serum of the immunized animal only after careful and continued immunization, usually later than the demonstrable appearance of the bactericidal or agglutinating properties of the serum. The most convenient material for such immunization consists of salt solution emulsions of agar cultures, killed at 60° to 70° C. These may be injected subcutaneously, intraperitoneally, or intravenously, the last method leading to the most satisfactory and rapid results and, therefore, best employed unless great inherent toxicity of the particular bacteria contraindicates. When rabbits are used it is generally necessary to inject 3, 4, or 5 times at 5- or 6-day intervals, and to bleed the animals on the 8th or 9th day after the last injection.

Specificity.—The bacterial precipitins so produced are, as we have said above, specific—but, again, specificity, as in the case of agglutinins, is limited by the so-called "group reactions." In the chapter dealing with agglutination we have seen that the serum of a typhoid-immune animal which agglutinates typhoid bacilli strongly will also agglutinate, though far less powerfully, paratyphoid bacilli and, in some cases, even colon bacilli, this appearance of "minor" agglutinins being probably due to a close group relationship of these bacteria to the typhoid bacillus. In the case of bacterial precipitins the same

[13] Myers. *Centralbl. f. Bakt.*, Vol. 28, 1900.
[14] Michaelis. *Deutsche med. Woch.*, 1902.
[15] Norris. *Jour. Inf. Dis.*, Vol. 1, 1904.

thing is true, and has been made the subject of special studies by Zupnik,[16] Kraus,[17] Norris,[18] and others. As in the case of agglutination, however, this fact does not in any way interfere with the practical value of the specificity of the reaction because elimination of the secondary group reactions, which in agglutination is obtained by dilution of the antiserum, can here be obtained, as Kraus points out, by diminishing the quantity of the undiluted precipitating serum added to the bacterial filtrates. Thus, while one volume of serum added to one, two, or three volumes of culture filtrate may still give error due to non-specific group reactions, a proportion of one part of serum to 8 or 10 parts of the filtrate will usually eliminate all secondary reactions and prove strictly specific.

An illustration of such an elimination of "partial" or "minor" precipitins by diminution of the amount of the homologous antiserum is given in the following table taken from the work of Norris [19]:

ANTICOLI RABBIT SERUM

TABLE III

The precipitating action of the anticoli rabbit serum upon its corresponding filtrates and upon the filtrates of B. N° 1 (hog cholera) and *B. typhosus*.

Coli filtrate	Anticoli serum	
0.5 c. c.	0.05	Cloudiness in all tubes in 1 hour at 37.5° C. which increases rapidly. Six hours well-marked precipitation—most copious in tube containing 0.25 serum. Fluid in all tubes becomes clear.
0.5 c. c.	0.10	
0.5 c. c.	0.15	
0.5 c. c.	0.25	
B. N°1 filtrate	**Anticoli serum**	
0.5 c. c.	0.10	At 6 hours a slight precipitate in the form of fine granules appears on the sides of the tubes. After 24 hours the precipitate in the tube containing 0.25 c. c. serum compares in amount to that formed in the homologous filtrate with 0.05 c. c. of serum.
0.5 c. c.	0.25	
B. typh. (Coll) filtrate	**Anticoli serum**	
0.5 c. c.	0.10	Similar reaction obtained to that with B. N° 1 filtrate.
0.5 c. c.	0.25	
B. typh. (Pfeiffer) filtrate		
0.5 c. c.	0.10	Similar delay in reaction as obtained with B. typh. Coll.
0.5 c. c.	0.25	

And, indeed, though the great practical value of the precipitin reaction has not been in the special field of bacteriology, it has been

[16] Zupnik. *Zeitschr. f. Hyg.*, 49, 1905.
[17] Kraus. *Wien. klin. Woch.*, 1901, No. 29.
[18 and 19] Norris. *Jour. Inf. Dis.*, Vol. 1, 1904, p. 472.

successfully utilized in the diagnosis of glanders by Wladimiroff,[20] and constitutes a valuable adjuvant to our methods of determining the biological relationship between micro-organisms.

The production of precipitins against unformed proteins, egg albumen, animal sera, etc., is much more easily accomplished than the production of bacterial precipitins, and three intravenous injections of from 2 to 5 c. c. of the protein at 5- or 6-day intervals usually give rise to a formation of potent precipitins. When a small quantity of the serum of such an animal, taken 9 or 10 days after the third injection, is mixed in a test tube with an equal quantity of a dilution of the protein which was injected, turbidity and rapid flocculation will result. In tests of this kind, unlike the bacterial precipitin tests in which the delicacy of the reaction is ordinarily determined by diminution of the amounts of antiserum, the same object may be more conveniently attained by dilution of the antigen. Thus, in testing the precipitating potency of, let us say, the serum of a rabbit immunized with sheep serum, we would proceed by setting up a series of small tubes, each of which contains a constant amount of antiserum (precipitin), but a progressively diminishing amount of antigen in the same volume—i. e., in dilution with isotonic salt solution. The following example will make this clear:

Antisheep serum from rabbit		Sheep serum 0.5 c. c. of following dilutions:		Precipitation
0.5 c. c.	+	1:10	=	±
0.5 c. c.	+	1:100	=	+++
0.5 c. c.	+	1:500	=	+++
0.5 c. c.	+	1:1,000	=	++
0.5 c. c.	+	1:5,000	=	+
0.5 c. c.	+	1:10,000	=	—

In this test it will be noticed that the strongest concentration of the antigen (1:10) gave a relatively slight precipitation only. This phenomenon is analogous to the inhibition zones noticed in the case of agglutination and other antibody reactions and will be more especially discussed in a succeeding paragraph.

The delicacy of the above example, moreover, is by no means unusual, and sera have been obtained by careful immunization with which the specific antigen could be detected in dilutions as high as 1 to 100,000 (Uhlenhuth). A serum which will react with antigen dilutions of 1 to 10,000 and 1 to 20,000 is not at all uncommon nor difficult to obtain. Apart from the additional advantage of the

[20] Wladimiroff. "Kolle u. Wassermann Handbuch," article on "Glanders," Vol. 5, 2d Ed.

specificity of the reaction, therefore, this biological method of detecting proteins is more delicate than that of any of the known chemical methods; neither the Biuret nor Millon's reaction will far exceed a delicacy of 1 to 1,000. By a modification known as the method of Complement or Alexin-fixation, furthermore, the delicacy of the biological reactions can be still further enhanced. This is discussed in detail in another place.

The practical value of the precipitin reaction, however, lies, not in the mere detection of protein, but, by virtue of its specificity,[21] in the determination of the variety of protein under examination. And here again the specificity, like that of bacterial precipitation, agglutination, and other serum tests, is relative rather than absolute. Thus a serum which has been obtained by the immunization of an animal with human serum may react, not only with human serum, but also with relatively higher concentrations of the sera of some of the higher apes. However, such non-specific partial reactions can be eliminated entirely by employing higher dilutions of antigen. Thus Uhlenhuth,[22] on the basis of a large experience, has established a standard of antigen dilution at 1 to 1,000, beyond which no "para" or "minor" precipitation will occur. Since potency far exceeding this is easily procured, absolute specificity can be ensured by the very simple precaution of a sufficient dilution.

Species Specificity of Precipitin Reactions and Its Uses.—The most important practical use for the reaction has been found in forensic medicine, where it is possible in this way to determine the species of animal from which have emanated the blood, sperm, etc., found in spots on wearing apparel, weapons, or other articles. The extensive investigations of Nuttall [23] upon this subject have incidentally been of much value in furnishing a further method for the determination of zoölogical species relationships. Nuttall carried out 16,000 precipitin tests, with precipitating sera, upon 900 specimens of blood which he obtained from various sources. He not only confirmed many of the accepted zoölogical classifications, but shed much light upon a number of disputed points. In working out the tests upon monkeys he found that the reactions carried out with antihuman serum become weaker as the species examined is farther removed from man zoölogically. Thus as we read down the column

[21] Wassermann and Schütze. *Deutsche med. Woch.*, 1900, Vereinsbeilage, p. 178; *Berl. klin. Woch.*, 1901; *Deutsche med. Woch.*, 1902; Bordet, *Ann. Past.*, Vol. 13, 1899; Nolf, *ibid*, Vol. 14, 1900; Fish, *Medical Courier*, St. Louis, 1900, cited from Wassermann.

[22] Uhlenhuth. *Deutsche med. Woch.*, 1900, 1901; *Rob. Koch Festchrift*, 1903. Uhlenhuth and Weidanz. "Kraus u. Levaditi Handbuch," etc., Vol. 2, 1909. Uhlenhuth and Weidanz. *Loc. cit.*, where other publications are summarized.

[23] Nuttall. "Blood Immunity and Blood Relationship," Cambridge University Press, 1904.

from man to the hapalidæ the precipitate becomes less and less in amount.

Nuttall's Tests with Antihuman Serum. (Nuttall, loc. cit., p. 165.)

ANTIHUMAN PRECIPITATING SERUM

Tested against	Precipitate
34 Specimens human blood	100%*
8 Simiidæ, 3 species	100%
36 Cercopithecidæ	92%
13 Cebidæ	78%
4 Hapalidæ	50%
2 Lemuridæ	0

* The percentages refer to the volume of precipitate formed on standing for a given time, the amount formed by the antiserum with its specific antigen being taken as 100 per cent. Antigen dilutions correspond throughout.

In another series he finds:

ANTIHUMAN PRECIPITATING SERUM

Tested against	Precipitate
Man	100%
Chimpanzee (loose precip.)	130%
Gorilla	64%
Ourang	42%
Cynocephalus mormon	42%
Cynocephalus sphinx	29%
Ateles	29%

Among the primates the highest figures with antihuman serum are given by the chimpanzee. Other bloods than those of the primates gave slight reactions or none whatever with the antihuman serum.

In addition to these results the relationships within the dog family, the horse family, and many other kinships similar to these were confirmed. In every case the precipitin reaction was consistent with the results of other methods of classification, and Nuttall's work is an extremely valuable aid to zoölogists in disputed questions of animal relationships.

These facts are the more surprising in that they demonstrate species differences between the proteins of various animals which are not determinable by known chemical methods. How fundamental these differences are and how delicate the reaction, is further shown by experiments of Uhlenhuth, in which he obtained a specific antihare serum by treating rabbits' with hares' blood, an astonishing result in view of the close zoölogical relations between these animals.

Isoprecipitins, that is, precipitins resulting from the treatment of animals with blood of another individual of the same species, have also been described by Schütze and others. They are not, however, regular in their appearance, nor are they very potent when obtained.

Since the reaction is equally applicable to vegetable proteins, similar investigations on the interrelationship of different varieties of wheat have been carried out by Magnus.[24]

The methods of performing precipitin tests for forensic or other purposes is extremely simple. Nevertheless, there are a number of theoretical considerations which we must take up in order to make clear the limitations of accuracy and conditions of control which are involved in these reactions. From our discussion of the nature of precipitinogen it follows that blood stains, etc., on linen or articles of any kind will be suitable for precipitin tests even after they have been exposed for considerable periods to unfavorable conditions, that is, an environment in which they are subjected to exposure to light, moderate heat, or drying. Thus blood spots, etc., if kept dry and in the dark, may give positive reactions even after years, as experiments by Uhlenhuth have shown. Meyer[25] claims even to have obtained a precipitation with extracts of the material of mummies. One of his specimens was a mummy dating back to the first Egyptian Empire (5,000 years), the other about 2,000 years old. Pieces of the leg and neck muscles of these specimens were chopped up finely, extracted for 24 hours with salt solution, then filtered until clear. With antihuman serum they gave turbidity after one hour at 37.5° C.

Under conditions of putrefaction, of course, the precipitinogen is more rapidly destroyed, though blood putrefies with surprising slowness, even if, as in our own experiments, the conditions of moisture, temperature, and reinoculation with putrefactive bacteria are constantly observed. Under such conditions a weak reaction may be obtained after as long as a month or six weeks.

In carrying out the tests with any material it is first necessary to get it into clear solution, a result which is best accomplished by soaking it in a small quantity of isotonic salt solution. Preliminary to this it is always necessary to scrape off a bit of the specimen and examine it microscopically to discover, if possible, whether blood cells, sperm, or other cellular constituents can be detected. The infusion in salt solution should be continued for several hours—if necessary for 12 to 24 hours. After the first few hours in the incubator the material should be placed at room or refrigerator temperature so that the yield in unchanged protein may not be diminished by the action of bacterial growth. After extraction the solution may be filtered in order to clear it, but often mere centrifugation suffices for this purpose. The concentration of antigen in such an extract is always an uncertainty, but may be determined with sufficient accuracy for practical purposes by shaking and observing the formation of a lasting foam. Protein solutions will show foam on shaking in dilu-

[24] Magnus. Cited from Uhlenhuth, *loc. cit.*
[25] Meyer. *Münch. med. Woch.*, Vol. 51, No. 15, 1904.

tions as high as 1 to 1,000, and if the original amount of salt solution used in washing out the material is properly gauged to the amount of blood available in the stain, and the solution shaken and observed for the formation of foam, it is usually a simple matter to obtain a final concentration approximating one to one thousand.[26]

The antiserum which is used should be of such a potency that preliminary titration with the specific antigen, diluted 1 to 1,000, should give an almost immediate cloudiness at room temperature.

By testing this serum against a number of other varieties of protein—dog serum, beef serum, etc.—it must be determined that the precipitin in this case is strictly specific.

The reaction can be observed with greater delicacy if it is first set up by the method recommended by Fornet and Müller,[27] which we may speak of as the "ring test." The antiserum is put into the tubes and the solution to be tested is allowed to flow slowly over this —as in Heller's nitric acid albumin test. At the line of contact between the two a fine white ring will rapidly appear, thickening and growing heavier as the preparation is allowed to stand. After taking the final readings from such a test, let us say after an hour or so, it is well to shake up the tubes, set them away in the ice-chest, and again read the amount of precipitates formed in the various tubes the next morning. Since every test of this kind necessitates a number of controls, the following example will serve as a basis for discussion:

Forensic Blood Examination

Material: Blood spot on trouser pocket, washed up in salt solution. Clear after paper filtration.

Antiserum: Rabbit treated with three intravenous injections, 2, 5, and 5 c. c. of human serum at six-day intervals; bled on tenth day after last injection. This serum has been titrated against human serum and gives precipitation in dilutions up to one to ten thousand. With one to one thousand there is clouding which begins in three minutes and is very distinct in eight minutes, at room temperature.[28]

Test

Tube 1. Known human serum 1 to 1,000	1.0 c. c. +	Antiserum....0.2 c. c.
Tube 2. Unknown solution to be tested	1.0 c. c. +	Antiserum....0.2 c. c.
Tube 3. Unknown solution to be tested	1.0 c. c. +	Normal rabbit serum.... 0.2 c. c.
Tube 4. Salt solution	1.0 c. c. +	Antiserum....0.2 c. c.
Tube 5. Unknown solution	1.0 c. c. +	Salt solution..0.2 c. c.

[26] If there is enough material, the amount of dissolved protein can be also approximately gauged by adding to a little of it a drop of acid, boiling and observing the heaviness of the cloud which forms. A control test of a known dilution of the suspected variety of blood can be made at the same time and the heaviness of this cloud compared with that in the test solution.

[27] Fornet and Müller. *Zeitschr. f. Hyg.*, Vol. 66, 1910.

[28] A mixture of too specific antisera should never be used, since such sera may often precipitate each other for reasons that are discussed below.

In this test, if the original material was human blood, tubes 1 and 2 should show ring formation within 5 minutes—while the other tubes remain clear. In addition to these controls it is well to be sure that the test extract is neither strongly acid nor alkaline, and that, as Uhlenhuth suggests, the material from which it is extracted does not contain other substances which can give precipitates by themselves when added to serum. This is especially necessary in the case of cloth fabrics, and a recent instance in our own experience has suggested to us the possibility that such materials may also contain colloidal dyestuffs or other extractable substances which can cause inhibition of the precipitation. In an apparently positive case the reactions with a blood extract from trouser cloth were sufficiently heavy, but regularly delayed, as in the flocculation of such colloidal suspensions as arsenic trisulphide in the presence of a protective colloid.

In the ordinary criminal or civil case which would come under consideration for precipitin tests the spots or stains are made by blood as it flows from the wound and unchanged by chemical or physical agencies except as these are encountered afterward, by exposure. In the case of meat inspection, in which the precipitin test is useful in detecting admixtures of horse flesh, dog flesh, or other less desirable varieties of meat, in sausages, chopped meat, etc., it often happens that such procedures as heating or smoking may vitiate the results of precipitin reactions. It is of practical importance, therefore, that we should know exactly what the effects of heating (boiling) may be upon precipitinogen. Moreover, this question possesses considerable theoretical interest since the coagulation of proteins by heat seems to involve chiefly a physical rather than a chemical change.

Cohnheim [29] says in discussing this question: "It is still unclear what the changes are that take place in coagulation. It may be that there is merely an intramolecular 'Umlagerung'—or there may be cleavage; or the process may be comparable to the flocculation of colloidal clay emulsions by salts. . . . With coagulation all proteins have lost the differences which they possess in the native state in respect to solubility or precipitability by salts. Physically all coagulated proteins are alike; they are no longer native proteins, and without further decomposition are insoluble. Chemical differences, however, variations of composition, and the cleavage products which they yield still distinguish them."

The question has been experimentally approached by Obermeyer and Pick [30] in connection with their general investigations upon the influence of chemical and physical alterations upon precipitinogen.

[29] Otto Cohnheim. "Chemie der Eiweiss Körper Vieweg Braunschweig," 1900, p. 8.

[30] Obermeyer and Pick. *Wien. klin. Woch.*, 12, 1906.

They found that precipitin produced with unchanged (native) beef serum does not react with heated beef serum, even if immunization was prolonged and a very potent serum was produced. On the other hand, when animals were immunized with beef serum which had been boiled for a short time ("Kurz aufgekocht" [31]) the precipitin so produced reacted, not only with native beef serum, but also precipitated the boiled serum and a whole row of split products which give no reaction to normal precipitin. The "coctoprecipitin" so produced, furthermore, was found by them to be specific, acting only upon beef protein or its derivatives.

It is immediately evident that these investigations are closely analogous to those of Joos and others on the agglutinins. The antiserum produced with the heated antigen here again reacts both with the native and with the heated antigen, whereas the antiserum produced with the native unheated antigen reacts only with the unheated. The "heat-precipitins" therefore may be also called "umfänglicher"—the term applied by Paltauf to the agglutinins produced with heated bacteria.

Schmidt,[32] who has studied the problem extensively, finds that heating serum protein to 70° C. for as long as 30 to 60 minutes alters its precipitability by "native precipitin" (precipitin produced by immunization with native unheated serum) only in so far as it diminishes the delicacy of the reaction by 10 to 30 per cent., and that heating to 90° C. for as long as an hour does not render it entirely non-precipitable, so that protein so treated may yet be detectable by ordinary specific precipitins produced by injections of unheated serum, though the delicacy of the reaction is lessened. Boiling, according to Schmidt, renders the antigen no longer precipitable by such "native precipitin," but, on the other hand, it does not seem to destroy its antigenic property of inciting precipitins on injection into animals. Fornet and Müller, on the other hand, claim that even boiled protein can be detected by "native precipitins," though the reaction is only about one-tenth as delicate as it is with unheated protein.

Schmidt studied these relations especially as they affect the performance of specific precipitin reactions in the identification of boiled meat. He found that when he immunized rabbits with serum protein that had been heated at 70° C. for 30 minutes the antiserum so obtained gave strong and practically useful reactions with its specific antigen even if this had been boiled. Since "native precipitin" gives weak reactions only with such a boiled protein,

[31] Sera or other proteins used in such tests are boiled in dilutions of 1 to 10 or more, in order to avoid the formation of heavy flakes which cannot be injected. Boiled in sufficient dilution, an opalescent suspension is formed which easily passes through the syringe.

[32] Schmidt. *Biochem. Zeitschr.*, 14, 1908; also *Zeitschr. f. Imm.*, Vol. 13, 1912.

Schmidt recommends the use of the "70° precipitin" (produced by injections of heated serum) for tests in which a heated antigen is to be identified.

He states, however, that very prolonged heating may so completely coagulate the antigen that none of it can be gotten into "solution" (suspension), and in such cases results can be obtained neither with the "native" nor with the "70° precipitin." He has attempted, therefore, to find a method whereby even such entirely insoluble proteins may be identified, and claims to have succeeded by preparing what he calls his "heat-alkali-precipitin." [33] He dilutes serum with equal parts of isotonic salt solution and heats it to 70° C. for 30 minutes in a water bath. To 60 c. c. of such a solution he now adds 10 c. c. of $\frac{n}{1}$ NaOH, and continues heating for 15 to 20 minutes. At the end of this time he neutralizes with HCl, cools, and injects 20 c. c. intraperitoneally into rabbits. (The neutralization is not absolutely necessary.) Five or more injections yield a serum sufficiently potent for use.

A precipitin so produced will, according to Schmidt, react specifically with heated proteins, and also with protein which has been solidly coagulated and brought into solution by means of NaOH and heat. It will not, however, react with normal unheated antigen.

He tested this by coagulating horse serum by boiling for 3 hours. The coagulum was washed with salt solution, dried, and powdered. Tests were then made to prove that this powder was entirely insoluble in NaCl solution. A little of it was then treated with 10 c. c. of salt solution containing enough NaOH to correspond to an $\frac{n}{1}$ solution. The exposure was continued for 20 minutes in a water bath at 60° to 70° C. Before the entire mass was dissolved the solution was filtered and neutralized with $\frac{n}{20}$ HCl.

The rather complicated relations described by Schmidt are easily surveyed in the following protocol taken from his work (see table I, p. 285):

Schmidt speaks of the "heat-alkali-precipitin" also as "alkali-albuminate-precipitin." It can be produced only if the NaOH treatment of the serum is cautiously performed. If the sodium hydroxid is allowed to act too vigorously in strong concentrations or for too long a time the antigen is completely destroyed, is no longer precipitable, and no longer produces precipitin when injected into animals.

The striking feature of these experiments is that they show a gradual alteration of the protein first by heat, then by alkali and heat, in such a way that the antigenic properties are changed but

[33] "Native precipitin" = precipitin produced by injections of normal unheated serum.

"70° precipitin" = precipitin produced by injections of serum heated to 70° C. for 30 minutes.

TABLE I

(W. A. Schmidt, *Zeitschr. f. Imm.*, Vol. 13, 1912, p. 173)

Solution of	Native precipitin	Heat (70°) precipitin	Heat-alkali-precipitin
Native serum	Strong reaction	Good reaction	0 (very slight turbidity)
70° serum (heated 30 min.)	Good reaction	Strong reaction	Strong reaction
100° serum (heated 30 min.)	0	Good reaction	Strong reaction
70° serum treated with NaOH (used to produce heat-alkali-precipitin)	0	0	Strong reaction
Boiled insoluble serum, brought into solution with NaOH	0	0	Good reaction
Native serum treated with NaOH in the cold	0	0	Good reaction

not destroyed. Each precipitin, moreover, seems to react most strongly with the particular antigen-alteration which produced it, and, according to Schmidt, retains its species specificity. This is not the case with the iodized proteins and nitroproteins and diazo-proteins produced by Obermeyer and Pick.[34] Here iodized beef protein injected into animals produced a precipitin which reacted with the iodized protein, not only of the beef, but also similarly altered proteins of other animals—and the same was true of the nitro and diazo modifications.

Although the experiments of Schmidt have great theoretical value, their practical utilization must depend upon the degree of specificity possessed by the heat-precipitins or the heat-alkali-precipitins. In Obermeyer and Pick's original investigations we have seen that they found the precipitin produced with heated serum as strictly specific as that induced by native serum. This has also been the experience of Schmidt. Fornet and Müller,[35] on the other hand, report that the precipitins produced by them with heated muscle-protein were not as strictly specific as those produced with the unheated—in that the former gave precipitates, not only with homologous protein solutions, but with foreign proteins in moderate concentration as well. In experiments carried out by the writer with Ostenberg [36] it was attempted to determine whether or not precipitins could be produced by injecting animals with protein that had been boiled, and if so what the action of these substances would be upon boiled proteins. Contrary to the results of Fornet and Müller,

[34] Obermeyer and Pick. *Wien. klin. Woch.*, No. 12, 1906.
[35] Fornet and Müller. *Zeitschr. f. Hyg.*, Vol. 66, 1910.
[36] Zinsser and Ostenberg. *Proc. N. Y. Pathol. Soc.*, 1914.

Experiments on Cocto-precipitin. Table II (March 23, 1913).

Cross titrations—dilutions of sera in salt solution boiled 5 minutes, precipitated with antisera produced by injections with similarly boiled material.

The readings here indicated were taken by "ring" test at the end of 30 minutes.

Dilution	Beef serum vs. anti-beef precipitin	Beef serum vs. anti-dog precipitin	Beef serum vs. anti-sheep precipitin	Dog serum vs. anti-dog precipitin	Dog serum vs. anti-beef precipitin	Dog serum vs. anti-sheep precipitin	Sheep serum vs. anti-sheep precipitin	Sheep serum vs. anti-dog precipitin	Sheep serum vs. anti-beef precipitin
1:20	+	+	+	++	—	+	++	++	+
1:50	+++	+	+++	++	—	++	+++	+	+++
1:100	+++	+	++	+	—	+	++	+	++
1:500	++	—	+	+	—	+	+	—	—
1:1,000	±	—	—	±	—	±	±	—	—
Controls of boiled serum alone*									
1:20	—			—		—			
1:50	—			—					
Serum control									

* These controls were necessitated by the fact that the boiled serum suspensions were themselves turbid and occasionally showed slight settling on standing.

it was actually found that sera boiled for 3 to 5 minutes injected into rabbits induced precipitins which acted upon boiled proteins, but at the same time it was determined that the antibodies so produced were no longer strictly specific. The protocol given at the top of the page will illustrate these experiments.

Summarizing these results together with those of Fornet and Müller and of Schmidt it would seem that the injection of boiled proteins induces precipitins which no longer act on native antigen, which act powerfully on boiled antigen, but are no longer strictly specific. This seems to us of great theoretical interest as showing an alteration by heating in the species adherence of the antigen. Practically, therefore, precipitins produced with boiled protein are of little value, and forensic determinations of boiled proteins should be done, as advised by Schmidt, by the "70° precipitins," or with native precipitin as practiced by Fornet and Müller.

Organ Specificity.—The specificity which is the basis of the practical value of the reactions that we have discussed is spoken of as "species" specificity since it has been found that the blood serum of rabbits or other animals into which the serum of another animal has been injected reacts, not only with the homologous blood serum, but also with extracts of the various organs of the particular species of animal which furnished the serum. Thus if we immunize rabbit, let us say, with sheep serum the resulting precipitin will react, not only with sheep serum, but also with extracts of sheep spleen, sheep

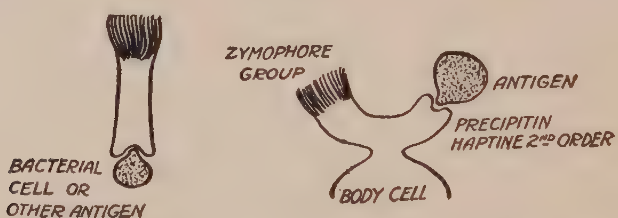
liver, etc. It seems that every species of animal possesses throughout its tissues a particular variety of protein, fundamental to its general metabolism and peculiar to its species. On the other hand, we have seen in the preceding discussions how chemically slight the changes in a protein may be which can alter materially its antigenic nature, and it is a logical deduction that different organs of the same animal might contain antigenic constituents qualitatively different from the general serum protein. There are undoubtedly in many organs protein complexes which are peculiar to them and not present in other organs, and it would be reasonable to expect therefore that immunization with separate organ substances would lead to the production of sera of specific precipitating power for the protein of that particular kind of organ. This is not ordinarily obtainable, however, because it has been impossible to isolate from organs their peculiar, characteristic proteins, and immunization of animals with organ extracts or solutions has necessarily implied the injection of much blood protein and other albuminous material of a character general to many organs of the animal, i. e., to the species. These quantitatively overshadow the organ-specific substances which may be present, and give rise, therefore, to a "species" precipitin. That "organ specificity," however, is a fact has been shown by the experiments of Uhlenhuth with the protein of the crystalline lens of the eye. Immunization with this substance induces a precipitin which does not react with the serum of the animal from which the lens was taken, but *does* react, not only with the crystalline lens proteins of this species of animal, but also with crystalline lens proteins in general, though taken from another animal species. Analogous to this are the experiments of von Dungern and others upon the protein derived from the testicle.

In both of these cases, as well as in other less sharply defined examples, the specificity is attached, not to the species of animal, but rather to the nature of the organ from which the particular protein is derived. These facts—first ascertained by means of the precipitin reaction—have been recently confirmed by means of the reaction of anaphylaxis by Uhlenhuth and Haendel, and by Kraus, Doerr, and Sohma. (See chapter on Anaphylaxis.) They have been discussed, moreover, in connection with the problem of specificity in general.

Biologically they probably signify that, although there are fundamental species differences between the general body proteins of various animals, there are still, in certain highly specialized organs, varieties of protein which, possibly because of functional exigencies, have developed similar chemical characteristics. These have been determinable by our present methods, however, only for organs like the lens, the testicle, and the placenta from which the organ-specific protein can be gotten in a relatively pure state. The pathological

importance of these phenomena lies in the fact that, although guinea pig serum injected into a guinea pig will not give rise to antibodies, lens protein apparently will do so—an observation which opens the possibility of autocytotoxins. The significance of this is indicated in such investigations as those of Römer,[37] who, using the complement-fixation technique to determine antibody, found that the serum of adult human beings possessed antibodies for their own lens protein, but that such antibodies were absent in the sera of children.

The study of agglutination and that of precipitation reveal, throughout, a close similarity between the two reactions, and indeed in physical principles they are probably the same, although the one (agglutination) consists in the flocculation of large particles in suspension—the bacteria—while in the other the precipitation is one of smaller units—the precipitable colloidal particles of the protein solutions. This phase of the subject will be more thoroughly discussed directly.



Schematic Representation of Ehrlich's Views on the Structure of Precipitins.

Ehrlich's Conception of Precipitins.—Meanwhile, it is noticeable also that, even without drawing the physical parallel between the two reactions, there is much in the behavior of the antibodies—the agglutinins and the precipitins as conceived by Ehrlich, which led him and his school to attribute to them a similar receptor structure. Like the agglutinins, the precipitins are not inactivated by 56° C., but when once rendered ineffectual by higher temperatures (70° C. or over) they can no longer be reactivated by the addition of fresh normal serum. For this reason chiefly Ehrlich attributed a similar structure to both agglutinins and precipitins, calling them "haptines of the second order."

Ehrlich assumed that when dissolved protein substances—ordinarily suitable for body nutrition—are injected into animals, they become anchored to the cells by such receptors of the second order. When overproduction occurs in response to repeated stimulation of the cells by consecutive injections (see Side-Chain Theory), these haptines of the second order circulate as agglutinins or precipitins.

[37] Römer. *Klin. Monatsbl. f. Augenheilkunde*, Sept., 1906. Ref. from "Weichhardt's Jahresbericht," Vol. 2, 1906, p. 348.

Since they act without the apparent coöperation of alexin, he supposes that they carry within themselves the "zymophore," or ferment groups, by means of which the agglutination or coagulation is accomplished. It is this zymophore group which, it is assumed, accomplishes the digestion of the foreign protein before its assimilation, when these receptors are still parts of the living cell.

Thus the conception of precipitins is identical with that formulated by the same school concerning the agglutinins, and the deductions from these premises have been essentially similar. Thus, analogous to the conditions prevailing in agglutination, Pick,[38] and Kraus and v. Pirquet [39] have shown that when precipitating serum is inactivated by heat, and then is added to bacterial filtrates, it will prevent their subsequent precipitation by active precipitin. An illustration of this is found in the following protocol taken from the paper by Kraus and v. Pirquet (*loc. cit.,* p. 69).

(a) 5 c. c. cholera filtrate + 0.5 c. c. inactiv. (60°) cholera serum = no precipitate after 10 hours at 37° C.
After 10 hours add 0.5 c. c. active cholera serum = no precipitate.
(b) Omitted.
(c) Omitted.
(d) 5 c. c. cholera filtrate + 0.5 c. c. active cholera serum = after 10 hrs. typical precipitate.

From this it was concluded that heat may destroy the zymophore or coagulating group of precipitins, leading to the formation of "precipitinoids" which, like agglutinoids, may have a higher affinity for the antigen than is possessed by the uninjured antibody.

Subsequently there were opposed to these views the physical interpretations which have been outlined sufficiently in the section on Agglutination (see p. 267). In the case of precipitation the analogy between colloidal reactions and the serum phenomena is fully as striking as in the former, an analogy in the delineation of which the first credit belongs to Landsteiner,[40] and important further contributions have been made by Neisser and Friedemann, Porges, Gengou, and a number of others. As in agglutination and colloidal flocculation, the presence of salts (electrolytes) fundamentally influences the occurrence of precipitin reactions; and in both colloidal and precipitin reactions the relative concentration of the reacting bodies is paramount in determining whether or not precipitation takes place. In this connection the most frequently observed inhibition occurring in serum precipitations is that which is caused by an excess of antigen. An example of this is as follows:

[38] Pick. "Hofmeister's Beiträge," Vol. 1, 1902.
[39] Kraus and v. Pirquet. *Centralbl. f. Bakt.,* Vol. 32, 1902.
[40] Landsteiner and Jagic. *Münch. med. Woch.,* No. 18, 1903; No. 27, 1904; *Wien. klin. Woch.,* No. 3, 1904. Landsteiner and Stankovic. *Centralbl. f. Bakt.,* Vols. 41 and 42, 1906.

Sheep serum 0.5 c. c.		Antisheep rabbit serum	Precipitate
1:10	+	0.5 c. c.	—
1:50	+	0.5 c. c.	±
1:100	+	0.5 c. c.	++
1:500	+	0.5 c. c.	+++
1:1,000	+	0.5 c. c.	++
1:5,000	+	0.5 c. c.	+

This is entirely analogous to the inhibition which may occur when, let us say, a weak gelatin solution is added to a colloidal suspension of arsenic trisulphid; or blood serum is added to mastic or arsenic suspensions. In both cases inhibition zones appear which show that the relative quantities of the two reacting bodies are quite as significant as their chemical or physical constitution in determining the occurrence of flocculation. This, according to Bechold, Billitzer,[41] and others depends upon the fact that the reason for flocculation is one of electrical charge. One hydrosol—say arsenic trisulphid—can be flocculated by the oppositely charged colloidal aluminium hydroxid, but this will occur only when the quantitative relations are properly adjusted. If one or the other is in excess, no flocculation may occur, and, if subjected to a direct current, both colloids, though ordinarily wandering in opposite directions, will now wander in that of the one which is now present in the largest amount. We will not elaborate here upon the causes for this, since they have been indicated in the section on Agglutinins and in the section on Alexin-fixation where Dean's accurate analysis of the importance of the relative amounts of antigen and antibody for precipitin reactions are discussed.

This effect of quantitative proportions would explain not only the absence of precipitation in the presence of too much antigen, but also the converse phenomenon, already mentioned, that precipitation may be inhibited when the precipitin is in excess.

The fact that heated precipitating serum when added to its antigen not only does not cause flocculation, but may even prevent subsequent precipitation by active precipitin, also finds its analogy in colloidal reactions in the so-called protective colloids. Thus arsenic trisulphid may be protected from precipitation by gelatin, if a small amount of gum arabic is added, and the analogy has been brought even closer by Porges,[42] who showed that heated serum will protect mastic suspension from precipitation by normal serum. This observation of Porges is so closely similar to the results obtained by Kraus and v. Pirquet and others on the inhibition of precipitation by heated

[41] Billitzer. Cited from Bechold, "Die Kolloide, etc.," p. 79.

[42] Porges. Chapter on "Colloids and Lipoids" in "Kraus u. Levaditi Handbuch," Vol. 1.

precipitating serum that it would seem, on first consideration, effectually to refute the conception of "precipitoids."

However, it does not explain the specificity of such inhibition on the part of heated precipitating serum, as reported by Kraus and v. Pirquet, an observation which is one of the strongest arguments in favor of the derivation of the inhibiting factor from the specific precipitin (a precipitoid).[43]

This apparent specificity of the precipitoids we think can be explained on the same basis on which we have explained the so-called specificity of "agglutinoids," where we have attributed it to the colloidal protective action of the inactive protein materials carried into combination with the antigen by the specific precipitin.

In spite of the strong evidence in favor of the colloidal interpretations, such contrary evidence, brought forward by careful and experienced workers, must be borne in mind and positive acceptance of the colloidal explanations, however attractive, must be withheld until much further investigation has been done.

Another important and interesting phase of the study of precipitins is that associated with the occasional presence in the same serum of remnants of antigen and of precipitins which, though present side by side, do not unite to form precipitates. This condition is frequently seen in such sera as those produced by Fornet and Müller[44] for rapid precipitin production for forensic work, a method in which the foreign serum is injected into rabbits in large amounts (2 to 10 c. c.), on consecutive days, and the animals are bled 6 to 8 days after the last injection. That such sera contain both antigen and antibody is shown by the fact that, though clear when taken, they will show precipitation not only when mixed with dilutions of the antigen, but also when added to homologous precipitating sera.[45]

This phenomenon has been noticed by Linossier and Lemoine,[46] Eisenberg,[47] Ascoli,[48] and others, and has been extensively studied

[43] Although normal sera may gradually precipitate on standing, this takes place much more rapidly in precipitin-sera. The spontaneous precipitation of normal sera as well as of those under consideration is analogous to what Bechold and others call the "ageing" (altern) of colloidal suspensions, which, though originally stable, will eventually settle out, even in the presence of protective colloids.

[44] Fornet and Müller. *Zeitschr. f. biol. Technik u. Methodik,* Vol. 1, 1908.

[45] For instance, a rabbit was injected on three consecutive days with sheep serum. It was bled on the fifth day after the last injection. The serum was clear when taken, but a precipitate was formed when it was added to sheep serum and also when it was added to serum from another rabbit similarly treated and containing sheep serum precipitin.

[46] Linossier and Lemoine. *C. R. de la Soc. de Biol.,* 54, 1902.

[47] Eisenberg. *Centralbl. f. Bakt.,* 34, 1903.

[48] Ascoli. *Münch. med. Woch.,* Vol. 49, No. 34, 1902.

by von Dungern.[49] Gay and Rusk [50] have recently observed it in connection with the rapid method of precipitin production of Fornet and Müller, and have noted that such sera, although containing both antigen and precipitin, do not possess complement-fixing properties. According to Uhlenhuth and Weidanz,[51] the antigen may persist in the sera of protein-immunized animals, in demonstrable amounts, as long as fifteen days after the last injection, and it is constantly present during this period, but in progressively diminishing amounts, eventually disappearing.

We are thus confronted by the apparently paradoxical phenomenon of the presence in these sera, side by side, of an antigen and its homologous precipitin, incapable of reacting with each other, although each of them readily reacts with precipitin or antigen, respectively, when these are added from another source.

Many attempts have been made to account for this. A number of observers, notably Eisenberg, have concluded from extensive analyses of quantitative relationships, both of agglutinin and precipitin reactions, that these take place according to the laws of mass action. In consequence, in addition to the combined precipitin-antigen complex present in all mixtures of the two, there should also be present free dissociated fractions of each, in amounts dependent upon relative concentrations. This might explain conditions such as those described above.

Von Dungern, whose paper forms one of the most extensive studies of the phenomenon with which we are concerned, does not believe that precipitin reactions can follow the laws of mass action, and explains the simultaneous presence of precipitin and antigen in the same serum by assuming a multiplicity of precipitins. He believes that every proteid antigen contains a number of related partial antigens which give rise in the immunized animal each to a partial precipitin. In sera in which both antigen and precipitin are found side by side and free, he believes that the antigen is of a nature that has no affinity for the particular partial precipitin present with it. He says: "Auch hier handelt es sich nicht um zwei reaktionsfähige Körper, deren Verbindung aus irgend welchen Gründen unterbleibt, sondern um Substanzen, welche keine Affinität zu einander besitzen. Die betreffenden Kaninchen haben zu dieser Zeit noch nicht alle möglichen Teilpräzipitine gebildet, sondern nur einzelne derselben. Diese zunächst produzierten, nur auf bestimmte Gruppen der präzipitablen Eiweisskörper passenden Partialpräzipitine sind es, welche nach der Absättigung aller zur Verfügung stehenden zugehörigen Gruppen der präzipitablen Substanz in Serum nachweisbar werden.

[49] Von Dungern. *Centralbl. f. Bakt.*, 34, 1903.

[50] Gay and Rusk. "Univ. of Cal. Public. in Pathology," Vol. 2, 1912.

[51] Uhlenhuth and Weidanz. "Praktische Anleitung zur Ausführung, etc.," Jena, 1909.

Daneben bleibt aber ein anderer Teil der präzipitablen Substanz, der keine Affinität zu dem gebildeten Präzipitin bestizt, bestehen, solange bis ein anderes Partialpräzipitin von den Kaninchenzellen geliefert wird, welches sich mit Gruppen der in Lösung geliebenen Eiweisskörper vereinigen kann."

Zinsser and Young [52] have also studied these phenomena and have attempted to explain them on the basis of protective colloidal action. In considering the theories that have been advanced to explain these occurrences, the conception of mass action as accounting for the simultaneous presence of the two reacting bodies in the same serum seemed entirely incompatible with our own observations and with those of Gay and Rusk, that these sera do not of themselves fix alexin. Were the conception of the manner of union of these two reagents, according to the laws of mass action, representative of the true state of affairs, it would be necessary to assume the presence, in such sera, not only of the two reacting bodies free and dissociated, but also of a definite quantity of the united complex of the two, a state of equilibrium being established. If this were the case the sera should, in agreement with all experience on the phenomenon of complement fixation, exert definite complement-binding power. Moreover, it has not been experimentally shown that colloidal substances react in accordance with the laws of mass action as observed for simpler chemical substances.

As regards the opinion of von Dungern, this seemed incompatible with another occurrence, observed by many writers, namely, that such sera, although clear at first, eventually, after prolonged standing, *do* actually precipitate spontaneously; that is, the union of the precipitin and the precipitinogen *does* actually take place, but goes on with extreme slowness.

Now a notable and strange feature of this phenomenon is the fact that two such sera, both containing antigen and precipitin, but neither of them precipitating by itself, will precipitate each other when mixed. For this reason Uhlenhuth has advised against the use of mixtures of precipitin sera for forensic tests. For it is not unusual that precipitin sera, even when produced by the slow method, may contain traces of antigen, and this may lead to precipitate formation if such a serum is mixed with another homologous precipitin and thereby simulate a positive forensic test.

In seeking analogy for this serum phenomenon with the various colloidal suspensions, the problem consisted in protecting two mutually precipitating colloids by a third, and this in such proportions that the mixing of two such protected suspensions, each containing all three of the elements, would be followed by precipitation. This was obtained by the use of gum arabic, gelatin, and arsenic trisulphid. Thin emulsions of gelatin will precipitate arsenic tri-

[52] Zinsser and Young. *Jour. Exp. Med.*, 1913, Vol. 17.

sulphid suspensions. Small amounts of gum arabic will act as a protective agent, preventing the precipitations.

The amount of the protecting substance necessary to prevent precipitation in any one mixture varies apparently with every change in the relative proportions of the two. Thus a considerable number of mixtures of the three can be made which will remain stable for days, the actual and relative quantities of the three ingredients differing in each of the mixtures. When two such mixtures are poured together, in many cases precipitation will result, varying in speed and completeness, according to the particular quantitative relationship arrived at in the mixture.

An example of such an experiment follows:

Two solutions of colloidal arsenic sulphid were prepared, one containing 1 gm. per liter, the other containing 5 gm. per liter. With Kahlbaum's "Goldruck" gelatin a solution containing 1 gm. per liter was prepared. A solution of gum arabic was prepared which contained 10 gm. per liter, this being made stronger than the gelatin solution to avoid too great dilution in the final mixtures. The gelatin solution was prepared twenty-four hours before being used, as freshly prepared gelatin has but slight precipitating power for arsenic sulphid, this power appearing to increase greatly with the ageing of the solution.

For the purpose of demonstrating this analogy two protected solutions were prepared as follows:

Solution 1.—This consisted of 2 drops of gum arabic, 2 c. c. of gelatin, and 5 c. c. of the weaker arsenic solution.

Solution 2.—This consisted of 10 drops of gum arabic, 1 c. c. of gelatin, and about 4 c. c. of the stronger arsenic solution.

In each case the arsenic sulphid was added until there were signs of increasing opalescence or turbidity, this being done in order that the two solutions should each be as little overprotected as possible.

Portions of the two solutions were then mixed in equal proportions. In the course of a few minutes the mixture was noticeably more turbid than either of the original solutions. This turbidity continued to increase quite rapidly, and on the following morning after about sixteen hours of standing, the mixture was found to be completely flocculated out, while the original protected mixtures remained unprecipitated and showed about the same degree of opalescence as on the preceding night. The same condition of affairs was found to have persisted after five days. On the fifth day the less concentrated of the clear protected suspension began to settle out, and was completely precipitated within twenty-four hours. The other remained clear for four days more, but on the ninth day it began to precipitate slightly, the precipitation remaining incomplete.

In these cases it appears, therefore, that a complete analogy to

the observed conditions of the serum reactions has been found, and that all data observed in connection with sera in which antigen and precipitin are found side by side without reacting can be most simply explained on the conception of protective colloid action. Moreover, the chemical nature of the substances involved seems to add weight to this point of view.

These relations have been gone into here at some length, since they seem to us to possess considerable theoretical and practical significance. For it may be that the presence of a protective colloid may, by inhibiting the union of antigen and precipitin within the body, protect the animal from intoxication during the early stages of immunization when antigen and antibody are present simultaneously for longer or shorter periods. Were union between the two possible at such times in the circulation, an assumption necessitated both by the hypotheses of mass action and of multiplicity of precipitins, there would probably be an absorption of complement by these complexes, with, as shown by Friedberger, a consequent formation of powerful toxic products. (See chapter on Anaphylaxis.) It is not impossible by any means, therefore, that the injection of antigen in an animal in which such a balance has been established may lead to a sudden elimination of the colloidal protective action, union of the antigen and antibody, and, by the mechanism just outlined, anaphylactic shock.

The fact, moreover, that mere heating will change the precipitating action, which certain sera have on inorganic colloids, to a protective one seems to show that this latter function may justly be associated with delicate physical or chemical alterations of animal sera.

Furthermore, this point of view is strengthened by the fact that the mutual precipitation of sera, such as those described, takes place slowly, as does the mutual precipitation of two protected colloidal mixtures, in contradistinction to the more rapid precipitation which takes place when any of these sera is added to an antigen dilution, where the element of protection may be assumed to be practically eliminated by more extensively changed quantitative relations.

This point of view has lately been disputed by Weil, who has gone back to the older view of von Dungern, largely on the basis of precipitation experiments he carried out with crystallized egg albumen. Weil claims that if a pure protein, like crystallized egg albumen, is used for immunization, antigen and antibody are never found simultaneously in the blood stream. If this were true it would indeed constitute a very important contradiction of our point of view. In consequence Bayne-Jones carried out similar experiments in our laboratory, and found that even with the purest obtainable recrystallized egg albumen both antigen and antibody are at times demonstrable in the blood. He showed this both by precipitation and by comple-

ment fixation. We do not consider the question closed, however, because it is indeed true that when working with a purified protein it is more difficult to demonstrate the two substances in any quantity, than it is when the crude serum antigen is used. This may be, of course, due to the fact that pure egg albumen may be more rapidly assimilated and remains in the circulation for a less extensive period than does the crude antigen. However, further experiments in this direction will unquestionably clear the matter up because it is a simple question of fact amenable to experiment.

CHAPTER XI

ISO-ANTIBODIES

Early in their researches, Ehrlich and Morgenroth were led to speculate upon the possibility of the formation of lytic antibodies within the animal against its own tissue cells. It would be of the greatest importance to pathology, they pointed out, if it could be shown that an animal could produce hemolysins, for instance, against its own blood cells. Thus, if an extensive internal hemorrhage occurred from trauma or other cause, in the course of which considerable quantities of erythrocytes are subjected to disintegration and absorption, it is at least conceivable that specific "autohemolysins" might appear which would lead to a chronic destruction of the red cells, with consequent anemia. This form of reasoning, as we shall see, has been extensively applied in the case of the cytotoxins for the explanation of a variety of pathological conditions. Ehrlich and Morgenroth approached the question experimentally in their further work on the hemolysins in goat blood. They found that it was comparatively easy to produce hemolysins in one goat by treatment with the erythrocytes of other goats, *isohemolysins,* as they called them.

Although, however, the blood serum of such an immunized goat was strongly hemolytic, not only for the blood cells of the goats whose blood had been injected, but also for the erythrocytes of certain other goats (though not, as we shall see, for goats in general), it was never in any case active against this goat's own cells. Moreover, while the other sensitive erythrocytes could absorb the hemolytic antibody out of the inactivated serum, the insensitive corpuscles of the goat himself seemed to possess no affinity whatever for the lysin of his own serum; mixed with the serum they failed to absorb out the hemolysin. This was in no sense, therefore, an *autolysin.*

These experiments show a remarkable individual variation between the similar tissues of animals of the same species, since Ehrlich and Morgenroth were indeed able to show that the insensibility of the goat's own corpuscles depended upon a complete absence of receptors for the isolysin. For, to explain the lack of "autolytic" action of such a serum, two possibilities could be assumed. One, as above, that the corpuscles of the goat possessed no receptors by means of which the isolysin could be "anchored" or, second, that, although

such receptors were present, they were already satisfied, or saturated with the lysin in the blood stream. In the latter case it would be hard to understand why hemolysis had not taken place.

In order to completely disprove the latter possibility, Ehrlich and Morgenroth did not allow the matter to rest upon conjecture, but resorted to an ingenious method of experimentation which yielded a further important result, namely, the discovery that the injection of antibodies into animals may give rise to "anti-antibodies." They injected inactivated hemolytic serum into goats whose corpuscles were sensitive to its action, and found that an "anti-isolysin" was formed, which, mixed with hemolysin and sensitive corpuscles, prevented hemolysis. Injection of such an isolysin into the goat from which it had been obtained, however, did not yield anti-isolysin, and it was therefore reasonable to suppose that its tissue cells possessed no suitable receptors. This failure of the production of antibodies by an animal against its own tissue cell has been spoken of by Ehrlich as "Horror Autotoxicus."

These rather involved experimental data will be shown to have a more than purely academic value when we come to speak of the problems of cytotoxin formation, and although they seem to show that auto-antibodies do not form as a rule, exceptions to this generalization have been observed. The most notable of these is the observation of Landsteiner and Donath [1] made in connection with the condition of paroxysmal hemoglobinuria. It was found that in such cases, in which hemoglobinuria follows exposure to cold, the blood serum of the patient contains an "autohemolysin." If the blood of such a case is taken into oxalate or citrate solution, and allowed to stand at ordinary or incubator temperature, nothing occurs. If, however, such blood is cooled to 0° to 10° C. and then warmed gradually to the temperature of the body, rapid hemolysis occurs. In this case the "amboceptor" of the serum is apparently fixed or anchored by the blood cells only at a low temperature, the complement becoming active as the blood is warmed. Although Landsteiner's observations are undoubtedly accurate, it is likely that this mechanism does not explain all such cases. The writer has had occasion to examine carefully a number of clinically diagnosed cases of this sort with a partially successful "Landsteiner" phenomenon in one of them only. Other observers have, however, confirmed Landsteiner's observation in well-established cases of the condition.

Taking its departure from these early observations, the problem of iso-antibodies, in general, aroused a considerable amount of attention among serologists and pathologists because it revealed an unexpectedly wide range of possible antigenic differences in the proteins of similar cells in animals even of the same species. The practical bearing on pathology was obvious.

[1] Landsteiner and Donath. *Münch. med. Woch.*, 1904, p. 1590.

The peculiar facts unearthed by Ehrlich and Morgenroth [2] indicated specific differences between red blood cells of individuals in the same species (goats), which could only be recognized by the development of immune isolysins. Work on other species of animals has indicated that this fact has a broad significance and that similar differences between individuals of the same species occur in many, if not all, species of animals. Isolysins similar in principle to those of Ehrlich and Morgenroth were produced by Ascoli [3] in rabbits; by Todd and White,[4] in oxen; by Ottenberg, Kaliski, and Friedmann [5] in dogs; by Ottenberg and Thalhimer [6] in cats, and by Hada and Rosenthal [7] in chickens. In all these instances the isolysins developed showed the same peculiarities, namely, that they attacked the cells of certain individuals and left the cells of other individuals of the same species unharmed. Recent work on the isolysins occurring naturally in the human blood has thrown considerable light on the nature of immune isolysins.

The occurrence of iso-antibodies in human blood was first noted by Maragliano [8] in 1892, and a large amount of work was done before it became perfectly clear that the occurrence of the iso-hemolysins noticed by him was not a characteristic of disease. Analogous to isolysins, iso-agglutinins against blood were first described in 1901, independently, by Landsteiner [9] and by Schattock. Landsteiner studied 22 individuals whom he could divide into three groups with respect to iso-agglutinins. The three groups were designated by A, B, and C, as follows: In Group A, the sera agglutinated the corpuscles of Group B, but not of Group C; the corpuscles of Group A, however, were agglutinated by the sera of both B and C; Group B, the sera agglutinated the corpuscles of Group A, but not those of C; the corpuscles of Group B were agglutinated by the sera of Groups A and C. In Group C, the sera agglutinated the corpuscles of both the other groups, but its corpuscles were not agglutinated by the sera of either of the other two. This preliminary classification was slightly changed in 1902 by Decastello and Stürli who added a fourth group, the corpuscles of which were agglutinated by both the other groups, but the serum of which agglutinated none of the others. A

[2] Ehrlich and Morgenroth. "Uber Hämolysine," *Berl. klin. Woch.*, 1900, No. 21.

[3] Ascoli. *Münch. med. Woch.*, 1901.

[4] Todd and White. *Nature*, June 23, 1910.

[5] Ottenberg, Kaliski, and Friedmann. *Jour. Med. Res.*, Vol. 28, 1913.

[6] Unpublished personal communication.

[7] Hada and Rosenthal. *Zeitschr. f. Imm.*, 1913, 16, p. 524.

[8] Maragliano. *IX Kongr. f. Innere Med.*, 1892.

[9] Landsteiner. *Wien. klin. Woch.*, 14, 1901, 1132. Landsteiner and Richter. *Zeits. f. Med.*, 3, 1902. See also Ascoli, *Münch. med. Woch.*, 1901.

concise description of these groups was finally given by Jansky[10] in 1907, whose classification of the four definite groups is shown in the following diagram.

Sera

Moss →			4	2	3	1
↓	Jansky →		I $\alpha\beta$	II β	III α	IV O
4	I	O	—	—	—	—
2	II	A	+	—	+	—
3	III	B	+	+	—	—
1	IV	AB	+	+	+	—

Cells

Jansky I = 40 % more or less
" II = 35 % " "
" III = 15 % " "
" IV = 5 % " "

Jansky's "I" and Moss' "4" is spoken of sometimes as the "universal" donor.—for reasons explained in the text.

In 1910, Moss[11] who studied particularly the parallelism of iso-lysins and iso-agglutinins in human sera, at that time unfamiliar with Jansky's classification, reported observations which are essentially identical with Jansky's, but, unfortunately, for uniformity of

[10] Jansky. Originally published in the *Klincky Sbornik,* 2, 1907. Information obtained from abstract in Report of Committee of Soc. of Amer. Pathol. Article has been abstracted in the "Jahresbericht über Leistungen und Fortschritte auf dem Gebiete der Neurologie und Psychiatrie," 11, 1907, 1092.

[11] Moss. *Johns Hopkins Hosp. Med. Bull.,* 11, 1910, 63.

classification happened to designate his groups in a manner directly opposite to that of Jansky, in that Jansky's Group I becomes Moss's Group IV, and vice versa. Thus, the two classifications are alike, but Groups I and IV are reversed. We have indicated this difference in the table, and wish to call particular attention to the fact that all clinicians who utilize these facts for transfusion purposes must be perfectly clear in their minds concerning the classification which they are using. Failure to do this may lead to accident, evident from examining this grouping that the phenomena can be explained (as Landsteiner has suggested) if it is assumed that there are two agglutinins (α and β) and two corresponding agglutinogens present in the red cells (A and B). The blood of the first group possesses both agglutinins, but no agglutinogens, the blood of the second group possesses agglutinin α, agglutinogen B, the blood of the third group possesses agglutinin β, agglutinogen A, the blood of the fourth group possesses no agglutinin but both agglutinogens.

The correctness of this conception can be proved by experiments in which various agglutinins are absorbed out of serum by the cells of the different groups, a line of study which has been followed particularly by Hooker and Anderson,[12] and recently, again, by Gichner.[13]

These agglutinins are present in weak dilution only, being generally active in dilutions only of 1-15 to 1-30. They are separately absorbed from the serum by the suitable red cells (Hektoen).[14] Ottenberg noticed that they were inherited, and this was also shown in 1908 and in 1910 by von Dungern and Hirschfeld,[15] who further found that this inheritance followed the Mendelian law strictly. The agglutinogens are the unit characters. The agglutinogens apparently are present at an earlier embryonic stage than the agglutinins. The agglutinogen, or agglutinability of the red cells, thus, is usually present at birth, while the specific agglutinative power of the blood serum, or agglutinin, may be absent at birth, and may not appear until several months later.

The laws of inheritance in regard to the transmission of iso-antibodies in human beings have been studied extensively since von Dungern and Hirschfeld's first observations. Ottenberg,[16] particularly, has followed a considerable series of families, and recently made the suggestion that in a very limited way, medico-legal use might be made of the reaction. This would, of course, as he admits, be necessarily a limited use for reasons that will become obvious when

[12] Hooker and Anderson. *Jour. Immunol.*, 6, 1921, 419.
[13] Gichner. *Jour. A. M. A.*, 79, 1922, 2143.
[14] Hektoen. *Jour. Inf. Dis.*, 1907, p. 297.
[15] Von Dungern and Hirschfeld. *Zeitschr. f. Imm.*, 4, 1910, p. 53 and 5, p. 284.
[16] Ottenberg. *Jour. A. M. A.*, 77, 1921, 682.

we discuss its possibilities. Buchanan [17] discussing this suggestion, disputed Ottenberg's assertion on genetic grounds. But since that time the question has been submitted to Thomas H. Morgan [18] whose discussion in the recent lecture before the New York Pathological Society is so interesting an example of the application of genetic reasoning to problems of immunology, that we think it worth quoting at length from his paper.

"Since Ottenberg's statement has recently been disputed by Buchanan, from an entirely wrong interpretation of Mendel's principles, I should like to point out that on the Mendelian assumption of two pairs of factors, all the known results are fully accounted for. If we represent one pair of genes by A and a and the other pair by B

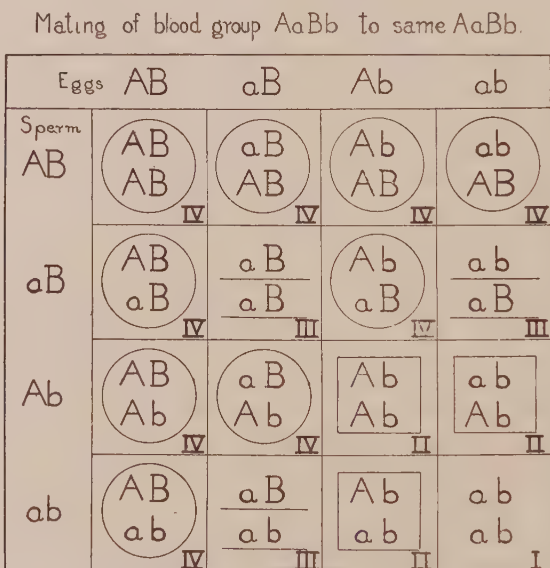


FIG. 8. Representing the kinds of individuals expected when an individual of the blood group type AaBb marries an individual of the same blood type, namely AaBb. Sixteen kinds of individuals are possible in the ratio of 9 : 3 : 3 : 1. These belong to four blood types, namely, class IV that contains at least one A and one B; class II that contains at least one A but no B; class III that contains at least one B but no A; and class I that contains neither A nor B.

and b, and if we represent an individual with the genetic constitution AaBb mating with another individual of like constitution (AaBb), then each will contain four kinds of germ cells, viz., AB, Ab, Ba, and ab. Thus sixteen possible combinations may be formed if any sperm may fertilize any egg.

[17] Buchanan. *Jour. A. M. A.*, 79, 1922, 180.

[18] Morgan, T. H. Goldsmith Lecture, *Rep. from Transac. N. Y. Pathol. Soc.*, 1922.

These sixteen individuals fall into four groups according to whether they have both A and B, or only A, or only B, or neither A nor B (i.e., ab) in the proportion of 9AB : 3A : 3B : 1ab. These four genetic classes correspond to the four recognized blood types IV, II, III, I, as indicated in the diagram. Now these sixteen kinds of individuals are found in all populations, so far studied, although in somewhat different proportions in different "races."

It is very simple to tell what the kinds of genetic offspring will be where any one of these sixteen individuals marries any other one. These possibilities are summarized in the following statement taken from Ottenberg:

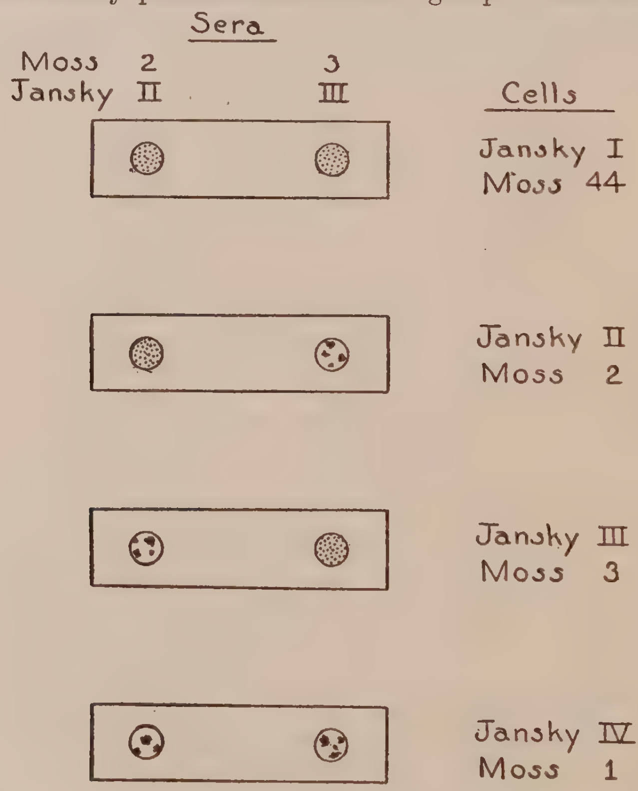
Unions of	I	and	I	give I
	I		II	I, II
	II		II	I, II
	I		III	I, III
	III		III	I, III
Unions of	II	and	III	give I, II, III, IV.
	IV		I	I, II, III, IV.
	IV		II	I, II, III, IV.
	IV		III	I, II, III, IV.
	IV		IV	I, II, III, IV.

From a knowledge of the blood group to which the child belongs it is possible to predict to what groups its parents *may* have belonged, and in certain cases it is possible to state that an individual of a certain group could not have been the parent of a particular child. This gives these considerations a practical medicolegal value which, it is true, is limited, but definite as far as it goes.

From the practical medical point of view, the importance of this subject is based on the increasing use of transfusion of human blood from one individual to another, especially in surgical practice. In selecting donors, it is, of course, desirable to select an individual of the same group as the recipient. Infusion from some of the other groups may bring about both agglutination, and as Hopkins first showed, phagocytosis of red blood cells in the circulation, with consequent injury to the recipient. In judging of the possibilities of harmless infusion, it is not necessary to memorize the combinations, but merely to remember that the chief danger occurs when the patient's serum is agglutinative for the donor's red cells, and a number of such transfusions have had fatal results. When the opposite occurs, that is, when the patient's corpuscles are agglutinated by the serum of the donor, the danger is far less, owing to the dilution of

the injected serum in the circulation of the recipient. Thus, in Jansky's classification where the serum of group I agglutinates the corpuscles of all the other groups, but the corpuscles of which are agglutinated by none of the other groups, group I could be regarded as a universal donor and used when a donor of the same type as the patient is not available. Conversely, this would apply to Moss's group IV.

For practical purposes, it is seen that tests can easily be carried out if one merely possesses stock sera of group II and III. The



following diagram illustrates this. Formerly these reactions were carried out with washed blood corpuscles in test tubes. This method has been greatly simplified of recent years by carrying out the entire test in drops on slides, a drop of a salt solution dilution of the blood of one individual being mixed with a drop of II or III serum, and observed for agglutination, either under the low power microscope, or with the naked eye. The reactions are prompt, sharply defined, and offer no difficulties, but precautions must be taken to prevent the drying up of the drops so that the increasing concentration of the salt does not bring about false agglutination.

Because of the confusion that the Jansky and Moss classification brought to the nomenclature, and the possible dangers accruing therefrom, a joint committee of the American Immunological and Bacteriological Societies took up the matter and after some study on the basis of priority, recommended a general adoption in the United States of the Jansky classification.[19]

The importance of iso-antibodies in such procedures as tissue transplantation, skin grafting, etc., has been variously studied with results which one might have expected, namely, that heterologous types did not lend themselves easily to such procedures. In general, this confirms our own observations on the toxicity of normal serum of one species to the red cells of another, in that it shows that a serum which has hemo-agglutinating or hemolytic action, also has a certain amount of action on other tissues of the body, being a general expression of incompatibility, not only of one type of cell.

Of the greatest interest in connection with iso-agglutination of red blood cells are studies recently made by L. and H. Hirschfeld.[20] Hirschfeld, working in Serbia, carried out thousands of iso-agglutination tests on different races. He found the agglutinable substances, A and B, present in all races, but a great preponderance of A in Europeans, and of B in Asiatics and Africans. Arabians, Turks, Jews and Russians were intermediate. The anthropological possibility of such an observation in pointing out the different racial intermixtures and the possibility of a double origin in Europe, is obvious.

Similar iso-agglutinins have been observed in the blood of lower animals, in horses (Klein,[21] 1902); rabbits (Boycott and Douglas,[22] 1910); cats (Ingebrigtsen); dogs, rats, and steers (Ottenberg).[23] The iso-agglutinins have been developed in dogs (von Dungern and Hirschfeld).[24] In most of the lower animals they have occurred with peculiar irregularity, indicating probably the presence of, not two, but of a larger number of agglutinins and agglutinogens. In steers, however, they fall into simple groups, indicating the presence of only one agglutinin and agglutinogen. In many animals the agglutinins are entirely latent, and the biochemical differences represented by the agglutinogens are present in the red cells, and the correct agglutinin is developed by the animal only when it is immunized with blood whose cells contain agglutinogen not present in the animal's own blood cells.

[19] Report of the Committees of the Amer. Assoc. Immunologists and Bacteriologists. *Jour. A. M. A.*, 72, 1921, 130.

[20] Hirschfeld, L. and H. *Lancet*, October, 1918, Vol. 2, p. 675, abstracted in the *Jour. A. M. A.*, 1919, 1641.

[21] Klein. *Wien. klin. Woch.*, 1902, p. 413.

[22] Boycott and Douglas. *Jour. of Path. and Bact.*, Jan., 1910.

[23] Epstein and Ottenberg. *Tr. N. Y. Path. Soc.*, 1908.

[24] Von Dungern and Hirschfeld. *Zeitschr. f. Imm.*, 1909, 1910, p. 531.

CHAPTER XII

A FURTHER CONSIDERATION OF THE NATURE OF ANTIBODIES

In the immediately preceding chapters we have considered the course of the occurrences which follow upon contact of the animal body with various infectious agents and their products. We have seen that one of the expressions of reaction in such cases is the appearance of the so-called specific antibodies which have the property of reacting with the particular bacteria or bacterial products which have incited them, in various ways, both within the animal body and in the test tube, and we have attempted to analyze such reactions. We hope, also, that we have made it plain that the phenomena with which we are dealing are by no means limited to the reactions of the body to bacteria, but represent general biological laws which govern the response of the living body to a large class of substances spoken of as antigens.

In the case of the antibodies formed in response to antigens of bacterial origin, the alterations in the physiology of the body of which antibody production is an expression, leads, under certain circumstances, to an ability of the body to defend itself against injury by bacterial poisons, and to remove the invading bacteria. In this process, circulating antibodies may, as in the case of the antitoxins, agglutinins and opsonins, be directly responsible for an important part of the protective process. Again, as we shall see in the chapters dealing with hypersusceptibility, the production of antibodies and their presence both in the cells which form them, or to which they become secondarily attached, and in the circulation, may lead to conditions of hypersusceptibility quite the reverse of protection in its practical consequences. The criteria which govern this are complex, and are considered in detail in the individual chapters dealing with the several antibodies and with anaphylaxis. Immunological analyses have, of necessity, dealt almost entirely with the consequences of antibody formation and the mechanism by which these consequences are governed, but have, up to the present time, left us almost entirely in the dark concerning the fundamental cellular physiology upon which antibody formation is based.

The fundamental source of all changes induced by antigen injections is, of course, the tissue cell.

It is reasonable to assume, as Ehrlich did, that *the tissue cells of higher animals are normally prepared only for the metabolic processes concerned in the nutritional and excretory functions essential to the maintenance of life,* the liberation of energy and the performance of any special secretory functions peculiar to their respective provinces of activity in the general body economy. Compared with the cells of the simpler forms of life, the normal functions of the mammalian tissue cells are considerably less wide in range. A protozoan cell, for instance, may take up boiled starch granules or bacteria and deal with them by complex processes, killing the bacteria in an acid vacuole, digesting them subsequently in an alkaline medium and extruding waste products. In only a few cells of the body, such as the phagocyting ones, either free or fixed, does such an atavistic ability to deal with substances unprepared by digestion survive.

We are not equipped to enter upon matters of cell nutrition in an authoritative manner, and, indeed, this is not necessary in the present connection. The essential consideration is that *in the bodies of the higher animals the substances which reach the cells for nutritional purposes through the blood or lymph stream, or perhaps through the intercellular fluids beyond the lymphatics, come into actual cellular contact only after elaborate preparation by preliminary digestive processes.* Thus, proteins probably reach the ultimate cells which they nourish only in the form of aminoacids; the fats are in the form of glycerol, fatty acids and perhaps soaps, and the carbohydrates in that of simple sugars. The cell is thus normally attuned only to dealing with substances in the chemical and physical conditions into which such preliminary digestion has put them. *It is only such substances, apparently, which can come into repeated or continuous contact with cells without in some way altering the quality or degree of the cellular reactions aroused by them.* It seems to be a fairly general biological principle that most materials which are not in this chemical and physical class of predigested nutritive matter give rise sooner or later to a specifically altered state of reaction capacity on the part of the cells. Thus, even when dealing with substances as relatively low in molecular structure as alcohol, some of the narcotics, quinine, morphine and other alkaloids, etc., the mere facts of habituation and occasional idiosyncracy indicate that such altered conditions have been produced. And, indeed, such drug tolerance or habituation cannot always be explained entirely by increased powers of excretion or destruction of the tolerated drug. For, although such increased eliminating properties are involved in the process, still it has been shown, at least in the cases of alcohol and morphine tolerance, that equal concentrations of the drugs in the blood stream of normal and of tolerant individuals may cause a deeper state of intoxication in the abstainers than in the habituated.

(Rubsamen, Van Dongen, cited from Wells.) Thus, it is necessary to assume, as Wells puts it, "a certain refractoriness or cellular immunity in addition." So, too, as we proceed upward toward the more complex substances with which the bacteriologist and immunologist more particularly deal, toxins and the proteins proper, contact of cells with these substances, incident to processes of infection or artificial parenteral administration, invariably induces an altered reaction capacity which is recognized either as some form of hypersusceptibility or of tolerance.

When we consider the varied chemical and physical constitution [1] of the many substances which may thus come into contact with the body cells, from the drugs at the bottom of the list to unchanged proteins, it need not astonish us if the manifestations accompanying the development of such susceptibilities or tolerances are subject to a wide range of variations. *Common to all of them, whether it is the fluctuating tolerance to morphine or the quasi-permanent acquired immunity to plague, cholera or typhoid, is a fact that the tissue cell is basically changed in its reaction to the particular material concerned* with a degree of *specificity which seems to be more striking as chemical complexity of the foreign substance increases.* Important differences, however, exist in the laws governing the acquisition of the altered cell reaction capacity and in the manifestations by which they may be recognized. These differences have been analyzed by a number of recent reviewers, and it is not necessary for our purposes to enter into the detailed controversial points involved.

Basic to such discussions has been the classification of all these phenomena into *two main subdivisions: Those in which definite antigen-antibody reactions* are involved and in which passive transfer to normal animals is, therefore, feasible; *and those in which no antibodies seem to be concerned and passive transfer is consequently unsuccessful.* The problem has received particularly careful analysis in relation to reactions of hypersusceptibility. Here the various manifestations of increased susceptibility to proteins in which antibody formation is unquestionably and easily demonstrable constitute a sharply defined class, the so-called condition of anaphylaxis, about the basic mechanism of which there is very little essential difference of opinion among workers. Apart from this well-defined group, however, stand many other phenomena of hypersusceptibility, in which the antibody mechanism so clearly concerned in typical protein anaphylaxis is either uncertain or can be definitely excluded.

The ability of the cell thus to express its response in the production of specific antibodies which, in the course of the reaction, may become free in the blood stream is the chief point which separates

[1] See Zinsser. Newbold Lecture, *Transact. Phil. College of Physicians*, 1922.

the mechanism of one type of reactions (the protein ones) from all the others. But even in reactions in which antibodies occur (immunity to plague, typhoid, etc., or anaphylaxis in the very early or very late stages after active sensitization) the changed reactions can exist on a purely cellular basis without the intervention of detached antibodies. Indeed, the cellular properties by virtue of which the typhoid convalescent remains resistant for years after recovery are just as little understood today as is the mechanism of the tolerance to morphine. *Regarded in this light, the formation of free antibodies, practically important as it is, may still be looked upon as an incident rather than as a fundamental difference,* dependent upon the manner in which the chemical or physical properties of the inciting substance permits it to react with the cell.

Ehrlich, whose lines of reasoning we have followed in the main, with others of his time conceived the cell as a *giant molecule,* a homogeneous chemical system with an enormous molecular weight, provided with a large number of "side chains;" these "side chains" were conceived as normally adapted for union with the various types of foodstuff that might be brought to the cell in the course of nutrition, but also capable of reacting with other substances reaching the cell under unusual conditions if they happened to have chemical affinities which fitted with one or another of the normally provided "side chains." It is not necessary for us to follow this well-known, purely chemical reasoning in his explanation of antibody formation. Since his time the *conception of the cell entity has changed considerably.* The material is perhaps most thoroughly brought together in Bayliss's book, and a clear discussion of some of the problems involved may be found in recent articles by Alsberg on chemical structure and physiological action. The cell can no longer be regarded as a large molecule of living matter, the life of which is maintained by a constant interchange of chemical unions and dissociations, but must be conceived, as Alsberg puts it, *"as a heterogeneous series of phases separated from each other by semipermeable surface layers,"* [2] resulting from the concentration at the surfaces, particularly, of lipoidal substances. But the semipermeable membranes which separate the internal subdivisions of the cell and delimit it from its environment probably do not consist of lipoids alone, but are composed of complex "colloidal intermixtures of lipoids and proteins." [3] It is clear that the reaction of an extraneous substance with a cell must depend not only upon its chemical but also upon its physical properties, according to which it may enter the cell and react with the substances in its interior or may come into direct contact only with

[2] See also work of Clowes.
[3] Bayliss. "Principles of General Physiology," Longmans, Green & Company, 1920.

the outer-surface phases of the protoplasm. It is also quite probable that the permeability of the cell membrane may change under various functional conditions, and that in the problems of hypersusceptibility a part of the process may consist of an increased ability of the injurious substances to get into the cell. In fact, though we have not yet made the matter one of special study, we have noticed on a number of occasions that the isolated uterus of a sensitized guinea-pig was more irritable to nonspecific extraneous substances than were the uteri of normal guinea-pigs. This matter, however, needs more definite investigation. *At any rate, the question of cell permeability to extraneous substances is quite as important in guiding our reasoning about immunological problems as is the matter of chemical structure.*

Alsberg points out three general ways in which substances may affect cells: They may attack the surfaces of the cells either by precipitating, coagulating or dissolving parts of the constituents of the surface, or by coming into chemical or purely physical union with only these surface layers. Again, they may enter the cell and cause chemical and physical alterations within the protoplasm. Other substances, again, may react with the cell in an indirect way only by altering the concentrations within the cell.

Thus, in reconstructing our ideas of the changes which may take place in cells under conditions of immunization or sensitization, we must depart from the purely chemical conception of Ehrlich to the extent of including in our considerations the physical properties of the foreign substances concerned, especially as regards their ability to enter the cells or to react with the surfaces only.

When we look upon the conditions governing altered cell reactions from this point of view, we are struck by the fact that *antibody formation, a property by virtue of which the inciting substance is classified as an "antigen," is exclusively an attribute of materials which are practically nondiffusible,* proteins or substances that have not been chemically separable from proteins hitherto. Considering this in connection with the fact that in test-tube reactions between such antigens and their antibodies the phenomena which are observed follow closely the analogy of colloidal reactions and are intimately dependent upon physical conditions of the environment, the thought suggests itself strongly that *nondiffusibility, the property of reacting only with cell surfaces, and antibody formation are in some way connected.* Although this thought is at the present time one that cannot be approached by direct experimentation, it is still of sufficient importance for the shaping of experimental thinking to be expressed. Thus, tolerance and hypersusceptibilities to drugs and other substances that pass easily through semipermeable membranes would be conceived as based on intracellular processes in which, perhaps, substances functionally analogous to antibodies may play a part,

but in which the ease with which the foreign substance can enter the cells renders unnecessary a mechanism for the production of circulating antibodies. In the case of proteins, on the other hand, where diffusion does not normally occur, the process takes place on the surface of the cell, and here the reaction products, as we know by observation, are eventually discharged into the circulation, and represent those factors in the circulating blood by virtue of which the tolerance or the hypersusceptibility can be transferred to normal animals.

The suggestion, thus, that antibody formation is in some way a consequence, not only of its chemical structure but also perhaps of the molecular size of the antigen, is one that must be borne in mind. Landsteiner came to this conclusion in a recent study of the chemistry of antigens, as we have from our own studies on anaphylaxis.

The Union of Antigen with Antibody.—In considering the various individual antibodies in the chapters on the toxin-antitoxin reaction, as well as in those dealing with agglutination and precipitation, we have seen that many different points of view have been expressed concerning the nature of the specific union. Ehrlich followed a purely chemical analogy in which atom groups of the tissue cell were assumed to have definite chemical affinity for atom groups of the injected antigen; that these cellular atom groups, when overproduced by the cell and discharged into the circulation, represented the antibody, and that, subsequently, therefore, the union of this antibody with the antigen was again based upon chemical affinity between definite parts of the molecules of each. According to Ehrlich's analysis, the reaction was supposed to take place by processes analogous to those by which a strong acid and a strong base unite. Quantitative irregularities in the curve of gradual saturation of one by the other Ehrlich explained on the basis of alteration products, such as toxoids, agglutinoids, etc., incident to which affinities of one for the other were altered. This conception of Ehrlich was modified by the school of Arrhenius, Madsen and their coworkers. While they admitted the chemical nature of the union, they explained the irregularities in the quantitative relations between the two, as fractional amounts of one were added to a unit amount of the other, by assuming that the antigen-antibody combination was dissociable or reversible, and followed laws of mass action, coming finally, in the case of each mixture, to a definite equilibrium.

Bordet and his followers, on the other hand, for reasons which have been set forth at some length in previous sections, denied that antigen-antibody unions under any circumstances took place by chemical laws of equivalents, asserting that the process was one of adsorption, comparable to that which occurs when any anilin dye is brought into contact with, let us say, filter paper. Bordet held that

the antigen-antibody union was in every way analogous to colloidal reactions in which the laws of adsorption, and, in this case, of course, specific adsorption, dominated the phenomenon.

It is not unlikely that it will become necessary in the future to alter all of these views, particularly on the basis of the recent work of Loeb.[4] Loeb's investigations upon the so-called colloids have led to a change in our conception of the reactions which take place between proteins and other substances which immunologists cannot afford to neglect. The adsorption theories applied to immunological processes, such as particularly those of Bordet, were largely based on analogy, and assumed that when protein reacted with other substances, colloidal or otherwise, the union was one in which the entire molecules of the reacting substances united. Loeb's work seems to indicate definitely that proteins may be regarded as amphoteric electrolytes which can exist in three states, according to the hydrogen ion concentration. Colloidal particles, as is well known, carry a definite charge which is dependent upon the hydrogen ion concentration. The particles carry a negative charge on the alkalin side of the so-called iso-electric point, and a positive charge on the acid side. For every particular colloidal suspension, there is a definite hydrogen ion concentration at which there is no electric potential difference between the particles and the medium in which they are suspended, so that they will not move in the electric field and are uncharged in their relationship to the media. Loeb has shown that at the iso-electric point, protein is in its purest condition, exists in a practically non-ionized condition, and is able to form neither metal proteinate nor protein acid salt. On either side of the iso-electric point, however, the protein combines like any other chemical compound, with acids, salts and perhaps other substances, in a characteristic way which is determined by the hydrogen ion concentration. Thus, in the case of gelatin, for instance, at which the Ph for the iso-electric point is 4.7, the hydrogen ion concentrations on the acid side of this combination can take place only with the anion of an electrolyte, forming, let us say, gelatin chloride. When the Ph is greater than 4.7, or on the alkalin side, it can unite with the cation only, forming sodium gelatinate, etc. Loeb conceives the protein molecule as presenting various atom groups for combination with materials with which it comes in contact, which, on the alkalin side of the iso-electric point, act something like fatty acids which are able to form salts with metals, etc., perhaps by combination with the COOH groups of the molecule. On the acid side of the iso-electric point the reverse is true, the proteins reacting like ammonia and being capable of uniting with only the anion of electrolytes, perhaps through their NH_2 groupings.

[4] Loeb, J. "Proteins and the Theory of Colloidal Behavior," McGraw-Hill, New York, 1922.

It is impossible for us in this space to go into the various proofs of such a conception of the reactions of proteins and the tremendous importance for the understanding of colloidal phenomena in general, which Loeb's conception carries with it. It is plain that it must alter and simplify considerably our points of view. It is, of course, as Loeb admits, quite possible that this is not the whole story, and that the difference in the behavior of proteins on the opposite sides of the iso-electric point may be accompanied by further intra-molecular changes in the protein molecule. However, this, until we know more about it, must remain undecided. There can be no doubt about the fact, however, that many of the substances which in immunology we have classified as behaving by the laws of colloidal reactions, must be regarded as amphoteric electrolytes and great attention must be given to the control of hydrogen ion concentration and the influence of cells, etc., in the reactions which are carried out with them. When we are working with bacterial suspensions, or suspensions of blood cells and the various normal and immune sera which are the instruments of immunology, we are using materials all of which come under the classification of colloids. As a matter of fact, one of the workers in our laboratory discovered by chance in following up this line of thought, recently, that practically all the ordinary serum reactions done in laboratories, were done at a Ph of at or about 8. It will be impossible in the future to ignore these facts in the performance of serum reactions and, especially, in quantitative work.

Moreover, the principles elucidated by Loeb have already indirectly led to what seems to us an important advance in our understanding of the union of antigen and antibody in the hands of Coulter.

Coulter [5] determined the movement of normal and sensitized red blood cells in an electric field and found, as others had before him with bacterial and other colloidal suspensions, that the movement of such cells in the field is the function of the hydrogen ion concentration. The iso-electric point for the cells he used was Ph 4.6. On the alkaline side they carried a negative charge and on the acid side a positive one. There is an old observation by Joos [6] that the salt which is necessary for the agglutination of bacteria did not act indirectly as supposed by some, but combined chemically with the bacteria. Joos using the minimum amount of salt necessary, found that it disappeared from the supernatant fluid; but until very recently this observation was neither confirmed nor further pursued. Coulter reinvestigated this in the light of Loeb's demonstration of the chemical nature of the relations of protein with acids, bases and salts, at various hydrogen ion concentrations, and found that, like colloidal protein solutions, both normal and sensitized cells combined

[5] Coulter. *Jour. Gen. Phys.*, 3, 1921, 309 and 513.

[6] Joos. *Zeit. f. Hyg.*, 36, 1901, 422.

chemically with inorganic ions. By adding decimolecular NaCl and HCl solutions, he found that the cells combined with the chlorine ions on the acid side of the iso-electric point, taking up amounts much greater than any they would take up on the alkaline side. On adding NaOH to such mixtures, he found that chlorin is actually given off by the cells. Conversely, he found that when he suspended the cells in isotonic barium chloride solutions, the barium ion was absorbed on the alkalin side of the iso-electric point. This behavior corresponded to that found by Loeb for his gelatin and other protein suspensions which combined with the cations on the alkalin side of the iso-electric point, and the anion on the acid side. He found, further, that the optimum agglutination for normal cells is at Ph 4.75, a point at which the cells supposedly carried no charge in relation to the surrounding medium, and are, if we follow out Loeb's views, in their most chemically pure form, and uncombined with inorganic ions. The optimum for the agglutination of sensitized cells was at Ph 5.3, a point probably related to the optimum for the flocculation of the immune bodies in the serum.

Of still greater significance are the further experiments of Coulter on the equilibrium between sensitizer and red cells in relation to hydrogen ion concentration. Working in a salt-free medium, namely, isotonic saccharose solution (9.2 per cent.), he measured the proportionate amounts of sensitizer which combined with the cells at various hydrogen ion concentrations, and, conversely, the amount of sensitizer which dissociated from the saturated cells when they were brought into a similar range of hydrogen ion concentrations. Both measurements corresponded with considerable accuracy, showing that in the combination a definite equilibrium is maintained between the two for each Ph. He found that the maximum combination between sensitizer and cells took place at or about Ph 5.3, the approximate iso-electric point of the serum globulin in which the immune bodies are probably carried, according to Rona and Michaelis.[7] At this point the combination between the two was almost 100 per cent. On both sides it diminished rapidly, on the alkalin side being reduced to not much more than 5 per cent. at a Ph of about 10. On the acid side it also diminished, but could not be measured to the same degree because of the hemolytic action of the increasing acidity.

The addition of sodium chloride greatly increased the proportion of sensitizer combining with the cells at all reactions except those near the iso-electric point where the union between the two seemed independent of the salt.

Apparently, if we follow Coulter in analyzing these facts in accordance with Loeb's observations, we can assume that the maximum union is at a point where almost all the immune body is present

[7] Rona and Michaelis. *Biochem. Zeit.*, 28, 1910, 193.

as an undissociated molecule not in combination with inorganic ions. The dissociated ions of the sensitizer formed either by its acid or basic dissociations, do not appear to unite with the cells, so that, as we pass from the iso-electric point in both directions, there would be a gradually smaller and smaller amount of undissociated, that is, unionized sensitizer present, and the union with cells would decrease. The salt solution, again in conformity with Loeb's observation, seems to depress the ionization, and, therefore, render the combination relatively independent of the Ph.

It need hardly be pointed out why a conception of this kind would render it imperative to take actively into account the Ph at which every antigen-antibody union or titration is carried out. It enforces a completely changed interpretation of the significance of the Bordet salt experiment, and definitely introduces the factor of calculable dissociation and reversibility into antigen-antibody reactions, making it possible to think of the reaction at every given Ph, as one at which an equilibrium is established.

The Dissociation of Antigen and Antibody.—In the preceding section we have gone into Coulter's recent work at some length because it seemed to us to follow out purposefully in immunological experiments, the newer conception of colloids and colloidal reactions which recent investigations have made necessary. The reversibility of the antigen-antibody reaction, however, has been the subject of many previous investigations. Landsteiner,[8] and Landsteiner and Jagic[9] in 1902 found that when red blood cells were agglutinated by abrin, and the agglutinated cells then rapidly washed in cold salt solution and finally emersed in a salt solution at 42° C., a certain amount of the abrin was split off from the combination and could be recovered. Experiments with normal agglutinins showed the same thing, the most successful dissociation of agglutinins from agglutinated typhoid bacilli being obtained at 55° C. At about the same time, Morgenroth[10] showed that when red blood cells were sensitized with hemolysin or amboceptor, and were then brought into contact with unsensitized cells, some of the hemolytic antibody was dissociated from the sensitized cells and became attached to the freshly added unsensitized ones. Similar observation of dissociation of antigen and antibody after union was subsequently made by a considerable number of observers. Bail and Tsuda,[11] Spaet,[12] Hahn and Trommsdorf,[13] Von Liebermann and Fenyvessy[14] digested

[8] Landsteiner. *Münch. med. Woch.*, 49, 1902, p. 1905.
[9] Landsteiner and Jagic. *Münch. med. Woch.*, 50, 1903, 764.
[10] Morgenroth. *Münch. med. Woch.*, 50, 1903, 61.
[11] Bail and Tsuda. *Zeit. f. Immunitäts.*, 1, 1909, 546.
[12] Spaet. *Zeit. f. Immunitäts.*, 7, 1910, 712.
[13] Hahn and Trommsdorf. *Münch. med. Woch.*, 47, 1900, 413.
[14] Von Liebermann and Fenyvessy. *Centralbl. f. Bakt.*, 47, 1908, 274.

sensitized pig corpuscles in N/100 hydrochloric acid in salt solution, precipitated the extracts with alkali, purified the precipitate with ether, and found that the final solution contained antibodies with too little protein to be determinable by qualitative tests. This may be cited as one of the earliest attempts to produce protein-free antibodies.

In 1918, Kosakai [15] washed sensitized sheep cells in saccharose solutions, and succeeded in recovering about five-sixths of the antibody combined with the antigen.

The most important recent contribution to this problem has been made by Huntoon.[16] Huntoon worked with various materials, but chiefly with the pneumococci. He has developed a method since then, by which he can dissociate a large proportion of the antibody from sensitized pneumococci by washing the agglutinated pneumococci with 0.5 per cent. sodium carbonate solution in salt solution. Such solutions could be repeatedly filtered without loss of antibody, and, therefore, obtained in a sterile manner. His antibody solutions did not give any of the ordinary qualitative protein reactions, were not affected by trypsin, and were not precipitated in solutions containing little or no electrolytes. They were not soluble in ether, and not dissolved by dilute alkalis or acids. They were still destroyed by temperatures over 60°.

With his so-called pure antibody solutions, Huntoon has been doing a considerable amount of clinical work which is not yet sufficiently analyzed for appraisal.

The results that he and clinical workers using his antibody solutions have obtained, indicate to us that, in addition to the antibodies, a considerable amount of bacterial material, possibly in the form of the residue antigens studied by us, is present in his solutions. From a theoretical point of view, his researches are of considerable importance in that they form a beginning of the possibility of our being able to study antibodies free from admixtures of inactive serum constituents.

Many of these investigations were made before we had a definite idea of the influence of hydrogen ion concentration on dissociation. With this knowledge available, it ought to be possible in the future to remove almost any desirable part of the united antibody by properly planned experiments.

Investigations such as these justify a certain amount of hope, therefore, that we may eventually be able to define chemically what the antibody consists in, but for the present none of this work has led to sufficiently accurate and detailed investigation to make it possible for us to come to a definite conclusion.

[15] Kosakai. *Jour. Immunol.,* 3, 1918, 109.
[16] Huntoon. *Jour. Immunol.,* 6, 1921, 117 and 123 and 185.

On the Essential Identity of the Antibodies.[17]—Since Ehrlich's [18] first classical analysis of antibodies, it has been a generally accepted conception of immunity that agglutinins, precipitins, sensitizers, bacteriolysins, hemolysins, or the so-called amboceptors, opsonins and the anaphylactic antibodies are separate substances formed in the animal body, often in response to treatment with a single antigen. Kraus,[19] in the first edition of Kolle and Wasserman's Handbook, summarizes this point of view unambiguously in the following words:

"Just as the bacterial body contains a variety of different antigens, so we may assume that animal protein is made up of a large number of different antigenic elements. If the animal body is treated with such substances and finds corresponding receptors, there results the formation of a variety of qualitatively different antibodies. . . ."

When Gengou,[20] in 1902, noted that alexin or complement was fixed when a precipitating antiserum was added to its homologous antigen, he interpreted this as meaning that, in addition to precipitins, the antiserum contained other antibodies, the "albuminolysins." To be sure, Gay and Moreschi [21] showed that the fixation of alexin was chiefly a property of the precipitate which was formed, but this was regarded as signifying that the protein sensitizers were mechanically carried down during the precipitation. Ehrlich, commenting upon this in 1910, says:

"It seems reasonable to assume, in accordance with Gengou's first explanation, that the property of binding the complement is exercised by the albuminous bodies sensitized with a specific amboceptor and, just as when immunizing with cells, agglutinins and amboceptors are formed, so also when immunizing with dissolved albuminous bodies two kinds of antibodies are formed, precipitins and amboceptors."

To be sure the idea that agglutinins and precipitins might represent one and the same antibody has been more or less prevalent since their first observation. It originated, we believe, with Paltauf [22] and was expressed as a widely accepted view by v. Eisler [23] in a review of precipitin reactions published in 1909. But, in regard to identification of other antibodies with these two, there has been either a complete disregard of such a possibility or, when suggested, it has been thrown out of consideration because of the frequent and

[17] Zinsser. *Jour. Immunol.*, 6, 1921, 289.
[18] Ehrlich. "Collected Studies on Immunity," translated by Bolduan, 2nd Edit., p. 585.
[19] Kraus, Kolle and Wassermann. *Handb.*, 1st Edit., 4, 1904, 617 and 618.
[20] Gengou. *Ann. de l'Inst. Past.*, 16, 1902, 734.
[21] Moreschi. *Berl. klin. Woch.*, 37, 1905, 1181, and 1906, 100.
[22] Paltauf. Quoted from V. Eisler, *loc. cit.*
[23] V. Eisler, Kraus and Levaditi. *Handb.*, 2, 1909, 835.

complete lack of quantitative parallelism between the curves of the various antibody functions in one and the same serum.

The idea of such a possible identity, however, has cropped up again and again and, in our own work, has gradually forced itself upon us so insistently that we have thought it important to bring it forward again.

The fundamental idea was expressed quite definitely in 1908 by Bail and Hoke [24] who made an extensive study of the bacteriolytic, precipitating and agglutinating actions of normal beef serum and immune rabbit serum upon cholera spirilla. They clearly expressed the opinion that there were no separate bacteriolytic, precipitating or agglutinating antibodies in these sera; that the essential fact was the existence, in the sera exerting these effects, of a single antibody which united with the bacterial substances and that the various reactions which followed were the results of the conditions under which the different observations were made. Although a similar idea, on somewhat less valid evidence, had been expressed by Bürgi,[25] and although Bail and Tsuda [26] followed out the thought in subsequent publications, the view has made little actual headway, in spite of much corroborative, though scattered, evidence.

In order that there may be no ambiguity as to just what is meant by what we call the "unitarian" view of antibodies, let us begin by formulating it clearly.

By such a conception of antibodies, we do not, of course, imply a complex cell like, for instance, the typhoid bacillus can give rise to one variety of antibody only. There may be formed a specific sensitizing antibody against the major chemical constituent, and other sensitizers against other antigenic substances enclosed in the same cell body or contained in the same antigenic solution. But we do mean that, were we working with a single antigen, in a pure state, one variety of antibody only would be produced. This would be present in the form of a serum constituent specifically capable of uniting with the antigen. As a result of the union, the antigen is altered in its physical, and perhaps, to some extent, in its chemical behavior. The resultant reactions which may be observed with this sensitized antigen (agglutination, precipitation, complement fixation, bactericidal phenomena, bacteriolysis, opsonization or sensitizing effects in the anaphylactic sense) would be determined not by differences in the nature of the antibodies with which the antigen had united, but rather by the physical state of the antigen itself, the nature of the coöperative substances (alexin, leucocytes, tissue cells), and by the environmental conditions under which the observations are made.

[24] Bail and Hoke. *Arch. f. Hyg.*, 64, 1908, 313.
[25] Bürgi. *Arch. f. Hyg.*, 62, 1907, 239.
[26] Bail and Tsuda. *Zeit. f. Immunitäts.*, Orig., 1, 1909, 546.

Thus, if the antibody comes in contact with a very finely divided antigen, as in a bacterial extract or in, let us say, horse serum, if electrolytes are present and perhaps other necessary physical factors furnished by the presence of serum, etc., precipitation occurs.

When we are dealing with whole bacteria of relatively large mass and correspondingly small surface exposure, agglutination is the result, and quantitative parallelism with the precipitin reaction is not to be expected because of the much greater dispersion of the antigen in the latter test.

When alexin is present, complement fixation or hemolysis or bactericidal effects result, since the changes produced by the sensitization have not permitted union with the complement.

When there are leucocytes present the union makes possible the phagocytosis of the antigen, and when the antibody is absorbed by the cells of an animal, anaphylactic "sensitization" occurs.

As we have stated, the earlier opposition to such a view was largely based upon lack of quantitative parallelism between agglutination and precipitation curves on the one hand, and bactericidal or protective antibody curves on the other, and this in spite of the relative inaccuracies which biological measurements of this nature cannot fail to involve.

In appraising such objections, however, we must not forget that agglutination and precipitation are actually only secondary phenomena, after the union of antigen and antibody has taken place, and are dependent upon a great many environmental factors which may not, to the same degree, influence phenomena in which alexin, the leucocyte or the body cells of animals are involved. We need only to point out the frequently observed alterations and diminutions of the agglutination and precipitating powers of sera by heat. Heating antibacterial sera even to 56° to 60° C. will often materially diminish their precipitating effects for bacterial extracts, an observation which is entirely analogous to the influence of heating (to 60° to 70° C.) on the flocculating effects of serum for various colloidal suspensions, like arsenic trisulphide, etc. In addition to this, the flocculation reactions depend upon the presence and the concentration of electrolytes, upon reaction, upon mutual relations of concentration, and perhaps upon viscosity. Moreover, the suspension equilibrium of the sensitized antigen must to some extent depend upon the varying factor of the inactive serum constituents carried into the union with the antibody. For we know that in precipitation reactions the bulk of the precipitate comes from the serum, and yet relatively protein-free antibodies can be split off from such a complex (Gay and Chickering,[27] Chickering,[28] Huntoon,[29] conditions which prove that, in the union, much

[27] Gay and Chickering. *Jour. Exper. Med.*, 21, 1915, 115.
[28] Chickering. *Jour. Exper. Med.*, 22, 1915, 248.
[29] Huntoon. *Jour. Immunol.*, 6, 1921, 117.

inactive protein substance is carried along, which inevitably must influence reactions of flocculation. Indeed, Tulloch has suggested that these "presumably inactive constituents" may even protect the united antigen-antibody complex from flocculation, a conception which we believe explains the "agglutinoid" phenomena.

It is not to be wondered at, therefore, that agglutination and precipitation curves should not run parallel with the curves of other antibody functions. And it is worth noting that, in regard to such lack of parallelism, while it has frequently been noted that agglutinating and precipitating functions were often weaker than other antibody effects or even absent entirely in such sera, it has rarely been observed that they were powerfully and specifically present when other effects were lacking.*

In addition, however, to these purely quantitative objections to the "unitarian" conception, other arguments have been advanced, such for instance as those of Gay and Stone [30] who observed, with cholera extracts and cholera sera, that the formation of precipitates removed little or no lytic substance from the supernatant fluid. As against this, however, we have the experiments of Bail and Tsuda who found that from specific cholera precipitates, obtained as above and injected into the peritoneal cavities of guinea pigs, lytic and bactericidal antibodies were liberated and effective in producing a Pfeiffer reaction.

We must confess that such failure of quantitative removal of antibodies from the supernatant fluid, after agglutination and precipitation reactions had been carried out with these sera, was our own experience with typhoid serum, and there are certain points here that require further investigation. But when one considers, as we have stated above, that the amount of sensitizer necessary to bring about an agglutination or precipitation may be very slight, even in the presence of an excess of antigen, and that from such agglutinated or precipitated bacteria or bacterial extracts dissociation can take place; and if we then further consider the great inaccuracies coincident to the quantitative determination of bactericidal and bacteriolytic effects, such experiments lose much of their weight. One need only recall such experiments as those of Topfer and Jaffe [31] who found absolute lack of uniformity between plating tests and Pfeiffer phenomenon in determining bactericidal substances in one and the same sera. There are too many and uncertain secondary factors in all these purely biological methods to give one much confidence in any reasoning based purely on quantitative results.

More recently the growing interest in the purification of antibodies by dissociating them from antigen-antibody unions has again led

[30] Gay and Stone. *Jour. Immunol.*, 1, 1916, 83.

[31] Topfer and Jaffe. *Zeit. f. Hyg.*, 52, 1906, 393.

* See also work of Northrop and deKruif on Agglutination, in a preceding chapter.

to attempts to separate antibodies in the pure state. Landsteiner [32] in his early work on dissociation makes no particular point of this phase of the problem. Gay and Chickering found that protective antibodies could be extracted from specific precipitates with dilute sodium carbonate, but interpreted this as a mechanical absorption of the protective bodies during precipitation. Huntoon succeeded in sensitizing pneumococci, heavily, and then, by treating them with slightly alkaline salt solution at 55° C. dissociating from them protective antibodies in considerable concentration, and in solutions which are practically protein-free. With these antibody solutions he has been able to protect mice. It is not our function to go into the experiments of Huntoon more extensively, since these are all published or in the process of publication. The important point for us is the fact that these highly protective antibody solutions fail to agglutinate pneumococci in a large number of tests made by him, although they possessed distinct protective powers.

Having considered some of the more important objections, let us see what may be said in favor of the "unitarian" view apart from its simplicity.

The identification of agglutinins with precipitins should hardly require much argument since both are specific flocculation reactions between a serum and the same antigenic substance, depending upon analogous environmental conditions. That they should differ from each other quantitatively is to be expected from the fact that in one case the antigen is present in relatively large masses and in the other case it is finely dispersed.

That there is at least a strong likelihood that precipitins and bactericidal and protective antibodies may be identical is indicated by the experiments of Bail and Tsuda who dissociated bacteriolytic cholera antibodies from precipitates obtained with cholera extracts and normal beef serum.

Bail and Hoke obtained similar results not only with normal beef serum, but with immune rabbit serum, though in the latter case somewhat less sharply. The latter point is important because the conglutination question is involved in their experiments with normal serum for they obtained much better precipitates with the normal beef serum in its active state. However, since sensitization with an antibody is necessary for the conglutinin effect, the principle of Bail and Hoke's experiment in regard to the liberation of bacteriolytic sensitizer from precipitated cholera extracts remains the same.

Also the identity of precipitins and protective antibodies is very strongly suggested by the experiments of Gay and Chickering, inasmuch as they succeeded in extracting protective pneumococcus antibodies from specific precipitates, and the identity of the two is at least likely as mere mechanical carrying down with the precipitate.

[32] Landsteiner. *Münch. med. Woch.*, 49, 1902, 1905.

The probable identity of precipitins with complement fixing antibodies is indicated by the work of Dean [33] and by many observations of our own.

In 1912, Dean, analyzing the relationship between alexin fixation and precipitation, concluded that the proportions of antigen and antibody which favor rapid and complete precipitation do not favor complete alexin fixation. He did not believe that the two reactions followed a parallel course, but said that he thought they represented two phases of the same reaction, a "flocculation representing the first stage of a change that can be recognized in its early stage by complement fixation." In 1912 and 1913 we were engaged in a similar analysis from which we came to the conclusion that there was no need for assuming that the antibodies which were involved in the fixation of the alexin were essentially different from those that brought about the precipitation. Indeed, in his Croonian lecture of 1917, Dean elaborated this idea, and came to the same conclusion that we did, namely, that all the various so-called antibody reactions were attributable to one and the same specific substance in the serum. This lecture of Dean, published during our absence, was, unfortunately not in our hands when our own paper on the same subject was written, but though he approached the problem from a slightly different point of view, his conclusions were essentially the same.

Studying the relationship between precipitin reaction and complement fixation in the reactions between sheep serum and anti-sheep rabbit serum, like so many other observers, we found that amounts of antibody which did not precipitate would, nevertheless, produce complement fixation reactions in such mixtures and this, of course, is the basic principle of the forensic complement fixation, introduced and described practically by Neisser and Sachs.[34] In order to eliminate the possibility of non-specific mechanical carrying down of the complement fixing antibody, we carried out experiments with complement fractionation, and found that the fixation by the precipitates and by the supernatant fluids in such mixtures took place in the same way that fixation by sensitized cells occurs; namely, that the precipitates first fixed end-piece and mid-piece, secondarily. From this and other experiences, we expressed the conclusion at that time that "there is but one variety of specific sensitizer," and that "the visible precipitation is merely secondary, occurring because of the colloidal nature of the reacting bodies under quantitative and environmental conditions which favor flocculation." Furthermore, the experiments of the last ten years have shown again and again that the physical state, the formation of precipitates and the changes in sizes and perhaps in electrical conditions of the substances in serum reactions are intimately related to the fixation of alexin.

[33] Dean. *Zeit. f. Immunitäts.*, Orig., 13, 1912, 84.
[34] Neisser and Sachs. *Berl. klin. Woch.*, 1905, 1388.

The recently developed observations on the Wassermann reaction, the Vernes test and the Sachs-Georgi reaction now successfully employed in our laboratory are all evidence in this direction. Recently the writer with J. T. Parker observed a pneumococcus horse serum which gave powerful precipitation with the residue antigens prepared from bacteria by us and described in another place. This serum, however, gave no complement fixation with the same antigen, and since this was consistently so, it seemed to contradict our idea of the identity of the two antibodies involved. We subjected this to analysis, and found that the discordant results were due to a special interfering substance in the horse serum which prevented the fixation of complement even when an anti-pneumococcus serum obtained from rabbits, was employed, with which perfect complement fixation was obtained unless the horse serum was added. It was thus found, that the failure to fix complement on the part of the horse serum could not be interpreted as indicating two separate antibodies.[35]

That the anaphylactic passive sensitizing effect of sera was found to be quantitatively proportionate to the precipitin contents of these sera, was shown by Doerr and Russ [36] in experiments which seem completely to justify the identity of precipitating and anaphylactic antibodies suggested by Friedberger in 1908.

In regard to the dissociation experiments of Huntoon, some of whose solutions were sent to us by Dr. Huntoon, himself, we have, indeed, found that these solutions exert considerable protective effect upon mice and fail either entirely, or almost entirely to produce agglutination *"in vitro"* of the pneumococcus against which they protect.

This would seem a potent argument against the "unitarian" idea. However, it must be considered that the "protein-free" antibody of Huntoon is physically, and, therefore, in its agglutinating and precipitating functions quite a different substance from the original antibody in the unchanged serum, which in its native state is associated with the inactive pseudo-globulin substances which it carries down with it into precipitates. For, as we have stated before, it is of course known that most of the substance which comes down in precipitin reactions is derived from the immune serum. Moreover, by, to some extent, restoring the Huntoon antibodies to their original environment in the circulation of an animal, we were able to observe powerful agglutination. We repeated, with these substances, the experiment on the mechanism of serum protection against pneumococci in rabbits, carried out by Bull [37] in 1915. A rabbit of approximately 1200 grams was intravenously injected at 5 P. M. with about 8 c.c. of a broth culture of pneumococcus I of relatively low virulence.

[35] Zinsser and Parker. *Jour. Immunol.*, 1922.
[36] Doerr and Russ. *Zeit. f. Immunitäts.*, 3, 1909, 181.
[37] Bull. *Jour. Exper. Med.*, 22, 1915, 466, 484, and 24, 1916, 25.

At 10 A. M. the next morning the rabbit was very sick; a heart's blood puncture was done which showed numerous pneumococci evenly distributed through the blood stream. Ten cubic centimeters of Huntoon's material was injected intracardially in this animal, the needle was withdrawn and about two minutes later blood was taken from the heart and smeared. Similar punctures were done five minutes later and fifteen minutes later. As will be seen from the attached illustration, smears from the heart's blood taken two minutes later showed the pneumococci in the blood in clumps of varying sizes, with very few small clumps of two and three, and a very few individual organisms. But the large majority of the organisms were now in clumps of 10 or more members. Smears after five minutes showed a few clumps and a few individual organisms, and after fifteen minutes there were very few single organisms and no clumps, but it was then hard to find organisms, whereas before injection every field showed ten or more.

We did not succeed in thus restoring the agglutinative functions of Huntoon's materials in all cases. But the reason for this was revealed, we believe, by experiments carried out for us by J. T. Parker. She found that the Huntoon substances, produced by dissociating sensitized pneumococci in dilute sodium bicarbonate solutions at 55° C., had a reaction of Ph 8.8 to Ph 9.4. When powerfully agglutinating pneumococcus sera were subjected to similar treatment at similar reactions, their agglutinating powers were largely lost and but partially restorable by neutralization. It would seem that these various considerations should serve materially to weaken the validity of objections to the "unitarian" theory based upon the separation of antibodies by dissociation experiments.

Furthermore, observations recently made by Coulter [38] seem to us to have considerable bearing upon this question in indicating that agglutination and the sensitization to hemolysis of red cells, are both due to the action of one substance. Coulter found that "the optimum hydrogen ion concentration for the agglutination of sensitized cells (rabbit-antisheep sensitizer), in a salt-free medium, occurs at Ph 5.3 which corresponds with the optimal point for the precipitation of the serum-globulin itself." The optimum for precipitation of serum-globulin, in which the immune bodies are carried, is stated by Rona and Michaelis [39] as Ph 5.2 and the iso-electric point for typhoid immune bodies as Ph 5.4. This reaction is more alkalin than that which is optimal for the agglutination of normal sheep cells in saccharose solution, so that Coulter concludes, as far as agglutination is concerned, the behavior of sensitized cells is closely related to the properties of the immune serum. We quote directly from a personal communication from Coulter as follows:

[38] Coulter. *Jour. Gen. Physiol.*, 3, 1921, 309.
[39] Rona and Michaelis. *Biochem. Zeit.*, 28, 1910, 193.

"Furthermore, an equilibrium has been found to exist between the amount of hemolytic sensitizer free and that combined with cells, the amount which combines at a Ph of 5.3 being at the maximum, and approximating 100 per cent."

These observations indicate that the hydrogen ion concentration at which the agglutination of sensitized cells is most perfect corresponds with the iso-electric point of that part of the serum which makes the antibodies and also corresponds with the point at which the largest amount of hemolytic sensitizer is absorbed by the red cells. This, to our mind, would be strong evidence in favor of identity of the hemolytic sensitizer and the agglutinin.

We do not wish by any means to convey the impression that we consider the "unitarian" view as absolutely and rigidly proven. We do believe, however, that the denial of such a view necessitates the assumption that the injection of a pure antigen calls forth five or six fundamentally different reactions on the part of the tissue cells, a theory which would be justified only on the basis of incontrovertible proof.

If there is nothing further to be said in favor of the "unitarian" view, one might at least wait for further evidence before one tried to deny an existing view, however complicated. But there is much to be said in favor of it and evidence in this direction is accumulating.

We have believed in the probable truth of the "unitarian" view for a considerable number of years, with sufficient conviction to teach it as the most likely state of affairs. And while we cannot prove it in all the ramifications of the difficult experimental problems involved, we believe it has gone far enough certainly to throw the burden of proof upon those who still cling to the separation and the conception of separate structure for agglutinins, precipitins, bacteriolysins, etc.

FURTHER ATTEMPTS TO APPLY PHYSICAL MEASUREMENTS TO ANTIBODY REACTIONS

In a number of discussions in preceding chapters we have emphasized the fact that serum reactions are gradually being recognized as having more in common with so-called colloidal phenomena than with those taking place during the union of crystalloids. The fundamental physical principles underlying colloidal reactions are as yet pretty vague and the serologist especially (ourselves included), when he speaks of colloidal reactions is often groping in the dark. Nevertheless, even without such fundamental understanding we can recognize the close analogies which exist between the various serum reactions and those taking place between substances recognized by their attributes as colloids. We have sufficiently discussed this in

the chapters on agglutination and precipitation, and need not enlarge upon it here except in indicating how the gradual tendency to pay attention to the physical factors involved has led to the actual working out of serum experiments in which the reasoning from the beginning and the technique finally developed were physical rather than chemical. Perhaps the earliest phases of such work were those in which complement fixation was shown to occur when complement was brought together with non-specific inorganic and organic colloids. It is due chiefly to the work of Landsteiner and his associates that attention was called to such phenomena. Landsteiner with Stankovic showed that complement may be fixed by silicic acid, and Seligmann soon afterwards found that in the precipitation of mastic suspensions complement may be fixed. Much work has been done that shows that a variety of colloidal suspensions of known chemical constitution fix complement, probably by adsorption. Perhaps the most striking instance of such complement adsorption is that occurring in the Wassermann reaction. Here, as we know, complement is fixed by a combination of syphilitic serum and various lipoidal suspensions, which may be entirely non-specific in origin. When the reaction occurs, as Jacobstahl and, later, Bruck have shown, a precipitation occurs which can be seen in the ultramicroscope. Furthermore, this precipitation takes place more rapidly in the ice-chest than in the incubator, a principle upon which is based the so-called refrigerator method of performing the test, and which strikingly suggests that the reaction is an adsorption rather than a true chemical union. It is this precipitate which fixes the complement; whether or not this is due to quantitatively increased globulin or to purely physical change in the syphilitic serum, is a matter which we cannot discuss at present. Whatever it is, it is unquestionable that the availability of the antigen for the Wassermann reaction depends not only upon its lipoidal nature but also on its state of dispersion. Since it is not possible, as we have found, to make available antigens for this reaction with non-lipoidal substances, like mastic, gelatin, gum arabic, salicic acid, albumins, and a number of other substances, even when in dispersion more or less similar to that of the Wassermann antigen, it seems that the secret of the Wassermann antigen must lie in the fact that substances of the chemical and physical constitution of lipoids when brought into a definite state of dispersion offer surface tension conditions not easily obtained with colloids of another nature. It is, therefore, at least in our opinion at present, the physical condition of the Wassermann antigen which makes it available for the test, a physical condition which is secondarily dependent upon the chemical nature of the dispersed substance. The importance of the state of dispersion and therefore the surface tension properties is quite apparent from the fact noted by many Wassermann workers that a considerable difference in the fixing power of the antigen may

be obtained by in one case adding the salt solution to the alcoholic extract quickly, and in another case adding it very slowly, the two separate preparations showing, one a very transparent, the other a very turbid, condition.

The Bordet-Danysz phenomenon has already been discussed, but is another case in point. It consists of the fact that if to a definite amount of antitoxin a definite quantity of toxin is added, the result is one of greater toxicity in the mixture if the poison is added to the antitoxin fractionally than when the entire amount is added at once. An interesting difficulty of this phenomenon is the fact that such a reaction seems to force upon us the assumption that the toxin-antitoxin union is not reversible, whereas later work on immunization with neutralized mixtures of these substances seems to necessitate the assumption that within the body they are reversible.

The "Epiphanin" Reaction.—It is a consideration of these and many other apparently physical factors of immune reactions which has led experimenters to seek to utilize physical changes for practical serological purposes. One of the reactions resulting from such studies is that known as Weichhardt's Epiphanin reaction. Weichhardt noticed that when two solutions of toxin and antitoxin are brought in contact with each other on exactly horizontal glass plates, diffusion takes place between them much more rapidly than in controls in which one or the other, antigen or antibody, was lacking. He tested this at first on the horizontal glass plates by adding coloring matter to the two solutions and observing the diffusion currents. Later he developed a method in which he tried to determine such increased diffusion by means of a delicate chemical balance. His method, briefly described in his own words, was as follows: "To the two arms of scales, to the right and left, a little bell-shaped jar is attached into which dilutions of serum are placed, on the one side specific immune serum, on the other normal serum. These little bell-jars are closed at the bottom with 'Schleicher-Schüll' filter paper and are immersed into solutions containing antigen in salt solution. Through the filter-paper membrane diffusion takes place, and in observations carried on for from several hours to several days it can be determined that the scales sink on that side in which specific immune serum had been placed into the little bell-jar. In other words, the antigen solution which is contained in the salt solution diffuses more rapidly on the side on which the specific immune serum was present. In consequence, the weight of the little bell increases and a definite reading can be made." Similar observations have been made by other observers, Kraus and Amiradzibi doing the experiment as follows: they employed a U-tube in the connecting horizontal part of which there was a glass stopcock. Into one side they put diphtheria toxin with a little aqueous methylene blue, and into the other side antitoxin. For each experiment two controls were

set up, one with salt solution and the other with normal horse serum, and they noticed that after a definite period of time after the stopcock connecting the two had been opened, methylene blue could be observed to diffuse across in the tube in which antigen and antibody had been present and not in the controls. Many modifications of technique to demonstrate this principle have been made by Weichhardt himself, and since the reaction is not likely to find much immediate diagnostic application because of its great delicacy, we need not describe them at length but refer the reader to Friedemann's excellent description in the Kolle and Wassermann, second edition, Vol. III.

Similar in principle but by no means identical is the so-called Meiostagmin reaction, chiefly developed by Ascoli.

The Meiostagmin Reaction.—Ascoli and Izar [40] have attempted to work out a diagnostic reaction which depends upon an alteration of surface tension of a fluid when an antigen unites with its specific antibody. Ascoli in his first experiments worked with typhoid bacillus extracts and the sera of typhoid patients, and found that when the two suspensions were mixed a reduction of surface tension resulted after time for union between the two had been allowed.

They determined the reduction of surface tension by Traube's [41] method by the use of apparatus spoken of as the "stalagmometer." The principle of this method depends upon the fact that as surface tension is reduced the number of drops to a given quantity of fluid is increased.

Diluted serum of patients was mixed with diluted antigen, and the number of drops contained in one cubic centimeter of the mixture was immediately determined and again measured after the mixture had remained for two hours in the incubator at 37° C. An example of one of Ascoli early measurements is given in the protocol cited below.

Reduction of surface tension results when various antigens are brought together with normal sera, but this can be easily controlled by suitable dilution, and must be carefully taken into consideration in each individual case. Ascoli and Izar have applied this method to the diagnosis of tuberculosis, typhoid, and various other diseases, and have reported what seemed to them reliable results. So far experience with the meiostagmin reaction has not been very extensive; not all observers have been able to obtain results as apparently reliable as those of Ascoli and his collaborators. It is not possible therefore to express a final opinion regarding this method of investigation; it contains, however, an interesting principle which with more exact methods of measurement may well become very important in serum diagnosis.

[40] Ascoli and Izar. *Münch. med. Woch.*, Nos. 2, 7, 18, 22, 41, 1910.
[41] Traube. *Pflüger's Archiv*, Vol. 123, 419.

1 c. c. of serum of typhoid patient diluted to 1–10.

		Number of drops	
		Immediately	After 2 hours in incubator
1 c. c. alcoholic typhoid extract diluted to....	1 0/00	57.8 58.1	59.7 59.9
	1 0/000	57.5 57.5	59.4 59.6
	1 0/000	57.0 57.0	59.3 59.2
1 c. c. alcoholic typhoid extract diluted to....	1 0/00	58.1 57.7	59.7 59.6
	1 0/000	57.4 57.6	59.4 59.2
	1 0/000	57.0 56.9	59.2 59.4
1 c. c. alcoholic precipitate taken up in distilled water..............................	1 0/00	56.5 56.5	58.0 57.8
	1 0/000	56.5 56.6	57.5 57.4
	1 0/0000	56.7 56.5	57.4 57.5
1 c. c. in 1 0/00 alcohol in 1 c. c. 0.85 per cent. NaCl solution....................................		56.6 56.7	57.5 57.6

Colloidal Gold Reaction.—An important reaction, depending entirely upon the principle of colloidal precipitation, is the so-called colloidal gold reaction extensively used for the diagnosis of syphilis of the central nervous system. In 1901, Zsigmondi, knowing that protein substances will protect various colloids from precipitation by electrolytes, attempted to work out a method by means of which he could quantitatively estimate protein in solution by its protective effect. He worked with colloidal metals, using especially colloidal gold. He determined what he called the "Goldzahl" for various proteins, by which he meant the amount in weight of a protein which was just enough to protect from precipitation 10 c.c. of colloidal gold, of a percentage of 0.0053, in the presence of 1 c.c. of 10 per cent. NaCl. It, of course, had long been known that in various pathological conditions of the central nervous system the protein contents of the fluid varied. Thus, the reactions of Nonne, of Noguchi, etc., were

all aimed at revealing an abnormal globulin content of the spinal fluid. Lange, attempting to apply Zsigmondi's method to the quantitative detection of proteins in the spinal fluid, observed a curious reaction, quite the opposite of what he had set out to find. He observed that spinal fluid, especially of syphilitic cases, in which there was protein material beyond the normal, precipitated rather than protected colloidal gold. He also observed that the dilution at which such precipitation took place was more or less constant in syphilitic cases and might therefore be utilized for the diagnosis of such diseases as paresis, locomotor ataxia, etc., etc. The reaction was taken up and carefully studied by a large number of observers, among whom were Zaloziecki, Jaeger and Goldstein, Flesch, Kafka, Lee and Hinton, Miller and Levy, and many others. One of the most practical discussions and clear descriptions available for American and English readers is that of Miller, Brush, Hammers, and Felton. The chief difficulty of the test consists in the preparation of a proper colloidal gold solution.

Lange adopted the method used by Zsigmondi with slight modifications. He added 10 c.c. of a 1 per cent. solution of gold colloid and 10 c.c. of a 2 per cent. solution of potassium carbonate to a liter of carefully and freshly distilled water. The solution was rapidly warmed almost to a boil and just before boiling, while actively stirring 10 c.c. of 1 per cent. formalin solution is added. The solution has remained colorless up to this point but upon this addition should almost instantaneously become a deep, transparent red. There should be no iridescent or smoky "Schimmer." The utmost care in getting pure distilled water must be exercised and Jena glass must be used throughout.

Other methods are those of Eicke, who adds dextrose, and many workers, such as Flesch, Kaffge, Eskuchen, and others, have found great difficulty in making up the solution by Lange's technique or by any of the other methods described. Miller and Levy state that with Lange's method good solutions can always be obtained if the water is absolutely pure and the glassware is satisfactorily cleaned; later, Miller, with the collaborators mentioned above, found that this was not universally true. Also, these writers, as well as Eskuchen, found that occasionally solutions which appeared all right did not react, and special studies followed dealing with the technique of the preparation of the gold sol. Miller and his collaborators find that the reduction method of producing this solution is the best one, and proceed as follows. They use the following reagents: [42]

1. The Gold Solution:

$AuCl_3$—Merck's yellow crystals hermetically sealed in brown glass ampules	1 gram
Water triply distilled, up to	100 c. c.

[42] The descriptions are taken direct from the thorough papers of Miller and Levy.

This stock solution is kept well stoppered in dark glass bottles away from any bright light.

2. The Alkaline Solution:

Merck's Blue Label Potassium Carbonate (desiccated)..........	2 grams
Water triply distilled, up to................................	100 c. c.

3. The Reducing Agents:

a. Formaldehyde, Merck's 40 per cent. stock solution, highest purity	1 c. c.
Water triply distilled, up to...............................	40 c. c.
b. Oxalic acid, Merck's Blue Label, Crystals.................	1 gram
Water triply distilled, up to...............................	100 c. c.

Solutions No. 2 and No. 3 must be made up immediately prior to use.

4. Bichromate cleaner for glassware:

Potassium Bichromate, powdered............................	200 grams
Water, distilled, up to......................................	1500 c. c.
Sulphuric Acid conc.	500 c. c.

The potassium bichromate should be well dissolved before the sulphuric acid is added. If this solution is reserved for cleaning glassware only, it can be used repeatedly.

Great attention must be paid to the cleaning of glassware. They boil their Jena beakers in Ivory soap solution, then brush under hot tap water. After being rinsed for five minutes, hot bichromate and sulphuric acid is added for half an hour. The beaker is then emptied and washed in running water for five minutes, rinsed with distilled water and then in triply distilled water. Similar methods are used with other glassware. The chief errors are insufficient brushing, failure to get all bichromate out, and allowing beakers to dry in air before using.

In obtaining the distilled water, they first take ordinary distilled water which they then re-distil from Jena flasks. They wash out the collecting flasks with the first 200 c. c. of the second distillate, then collect and re-distil in the same way, again rinsing with the first 100 c. c. of the second distillate. After all these steps have been performed the rest is more or less simple. A beaker rinsed out with triply distilled water is filled to the liter mark and the temperature raised to 50° C. gradually, then the gas is turned on full and when the temperature has reached 60° C. 10 c.c. of the 1 per cent. gold solution and 7 c.c. of the 2 per cent. potassium carbonate solution are added. The solution should remain perfectly clear. At 80° C. while stirring with a clean thermometer, 10 drops of oxalic acid are slowly added. The solution may now turn a delicate bluish-pink, often due to an excess of alkali. Otherwise the solution remains colorless until 90° C. has been reached. When 90° C. has been reached, the gas is turned out and, while stirring, 5 c. c. of 1 per cent. formaldehyde is added, drop by drop. If a pink color makes its

appearance before all the reducing agent has been added, Miller advises to stop at once. He also states that the best solutions are those in which the color change is slow. For further technical points, the reader is referred to the paper of Miller and his collaborator in the Johns Hopkins Hospital Bulletin, 1915, xxvi, 391, from which this description is almost bodily taken.

The test is done as follows: into a clean test tube 1.8 c. c. of fresh sterile 0.4 per cent. NaCl solution is placed. Into ten further tubes 1.8 c. c. of the same salt solution is placed. To the first tube is added 0.2 c. c. of spinal fluid to be tested and well mixed. 1 c. c. of this 1:10 solution is added to the second tube, mixed, and of this 1 c. c. is added to the third tube, in consequence of which dilutions are obtained running from 1:10, 1:20, 1:50. Now to each of these is added 1 c. c. of the colloidal gold solution. Changes begin to take place in five minutes, which usually reach their completion in about a half hour. The spinal fluids must be free from blood and clear from bacterial contamination.

Normal spinal fluids produce usually no reaction. The so-called luetic curve is usually one in which the greatest precipitation occurs in the tubes ending in 1:40 to 1:160. In suppurative lesions, etc., the strongest precipitation occurs in higher dilutions, say, from 320 to 280, which Lange has called "verschiebung nach oben." A so-called paretic curve means complete precipitation in, say, from 110 to 160, with a gradual fading out toward the higher dilutions. A complete precipitation means a colorless tube; partial precipitation shades from pale blue to the complete red of the unaffected colloidal gold. Fluids from early stages of syphilis without nervous system involvement react usually like normal fluids.

That the reaction has unquestionably found a permanent usefulness cannot be doubted. It seems to be dependent entirely upon the technique of making the colloidal gold solution.

CHAPTER XIII

PHAGOCYTOSIS

Early investigations into the fate of bacteria within the infected animal body were largely carried out by pathological anatomists, and the observation of the presence of micro-organisms within the cells of the animal and human tissues was definitely made as early as 1870. Hayem,[1] Klebs,[2] Waldeyer,[3] and others, saw leukocytes containing bacteria but failed to interpret this in the sense of possible protection. The process was regarded rather as a means of transportation of the bacteria through the infected body, or it was assumed that possibly the micro-organisms had entered these cells because of the favorable nutritive environment thus furnished.

The first to suggest that such cell ingestion might represent a method of defence was Panum,[4] who referred to it as a vague possibility. A similar but more convinced expression of this opinion was made in 1881, according to Metchnikoff,[5] by Roser in explaining the resistance of certain lower animals and plants against bacteria. But Roser brought no experimental support for his contention, and little attention was paid to his assertion.

The significance of cell ingestion as a mode of protection against bacterial invasion, therefore, was hardly more than a vague suggestion when Metchnikoff, who, though a zoölogist, had become intensely interested in the problem of inflammation, began to experiment upon the cell reaction which followed the introduction of foreign material, living or dead, into the larvæ of certain starfishes (Bipinnaria).

Pathologists, at this time, held complicated views of inflammation which involved complex coördinated reactions of vascular and nervous systems, and Metchnikoff's primary purpose was to observe reactions to irritation in simple forms devoid of specialized vascular or nervous apparatus. He noted in these transparent, simple forms of life that the foreign particles were rapidly surrounded by masses of ameboid cells and reached a conclusion which, in his own words, is expressed as follows:

[1] Hayem. *C. R. de la Soc. Biol.*, 1870.
[2] Klebs. *Pathol. Anat. der Schusswinden,* 1872.
[3] Waldeyer. *Arch. f. Gynekol.*, Vol. 3, 1872.
[4] Panum. *Virch. Arch.*, Vol. 60, 1874.
[5] Metchnikoff. "L'Immunité dans les Maladies Infectieuses."

"L'exsudat inflammatoire doit être consideré comme une réaction contre toutes sortes de lesions et l'exsudation est un phenomène plus primitif et plus ancien que le rôle du système nerveux et des vaisseaux dans l'inflammation." [6]

He compared the process of cell ingestion or phagocytosis of foreign particles, as here observed, to that taking place in the most simple intracellular digestion which occurs in unicellular forms, a hereditary cell function now specialized in certain mesodermal cells, and passed on in the evolution of higher forms to other specialized cells. And indeed in animals of the most complex structure the leukocytes which carry on this phagocytic process may be considered as, in a way, representing a primitive form of cell, since they are only nucleated elements of the body which wander from place to place, and are anatomically independent of nervous control. In 1883, at the Naturalists' Congress in Odessa, Metchnikoff [7] first expressed his views and communicated the first of the splendid researches upon which our modern conception of phagocytosis is largely based.

His earlier studies were carried out with a small crustacean, the daphnia, in which he studied the reaction which followed the introduction of yeast cells. He observed the struggle which ensued between the ameboid leukocytes of the crustacean and the infecting agents and determined that complete enclosure of the yeast within the leukocytes assured protection to the daphnia, while a failure of this process, either from fortuitous causes or because of too large a quantity, or too high a virulence of the infecting agents, resulted in disease and rapid death.

This early work of Metchnikoff forms the beginning of a long train of investigations to which we owe most of the basic facts we possess concerning the rôle of the phagocytic cells in the protection of the body against infection. Just as the various serum phenomena, of which we have spoken, have a general biological significance apart from their importance in relation to bacterial invasion, so the process of phagocytosis must be looked upon as an attribute of the animal and vegetable cell which has important physiological bearing entirely apart from infection.

In fact, the ingestion of bacteria and other foreign particles by the leukocytes and other phagocytic cells of higher planets and animals is entirely analogous to the intracellular digestive processes which take place, as the ordinary manner of nutrition, among the unicellular forms. Among the rhyzopods, in general, food is taken

[6] Inflammatory exudation should be considered as a reaction against all sorts of injuries, and exudation is a phenomenon more primitive and ancient than are the parts played by nervous system and blood vessels in the process of inflammation.

[7] Metchnikoff. *Arb. a. d. zool. Inst.*, Wien, Vol. 5, 1883.

in by means of the ingestion of other smaller forms of life, bacteria, algæ, etc. (or particles of dead organic matter), into the cell body of the protozoön.

These materials are gradually engulfed by the body of the ameba, which flows about them with its pseudopods, and within the cytoplasm undergo gradual digestion. The process has been carefully studied by Mouton.[8] In symbiotic cultures of amebæ with colon bacilli on agar plates, the bacteria are taken up in large numbers and about them are formed small vacuoles. That the digestion takes place in a slightly acid medium with the vacuoles can be proved by adding a drop of neutral red to the hand-drop preparation of amebæ and observing the brownish-red color taken by the materials in the vacuoles. Mouton was able to obtain a digestive ferment from the amebæ, by glycerin extraction, which exerted strong proteolytic action upon various albuminous substances, liquefied gelatin, and digested dead colon bacilli in vitro, acting best in slightly alkaline, but also in slightly acid, reactions. It is plain, therefore, that the most primitive form of digestion is an intracellular one carried on by ferments comparable in every way to the secreted digestive enzymes which accomplish the same purpose outside of the cells in higher animals. In essence, however, there is no fundamental difference physiologically between intra- and extracellular digestions, and the intracellular manner of assimilating solid nutritive particles may be retained in forms much higher in the scale of evolution than the rhizopods. It has been studied by Metchnikoff and others in certain of the flat worms (*Dendrocelum lacteum*) in which typical phagocytosis is carried on by the cells of the intestinal mucosa. Many of these planaria obtain their nourishment by sucking the blood of higher animals. Placed under a microscope after feeding, it may be seen that the foreign blood cells are rapidly taken up by the intestinal epithelial cells, which engulf them by means of pseudopodia not unlike those of the ameba. After ingestion, here, too, the blood cells are surrounded by vacuoles within which their gradual disintegration or digestion is accomplished. Similar intracellular digestion seems to be general among the cœlenterates, and has been thoroughly studied by Metchnikoff in the actinia. Here the food particles are carried by the tentacles into the esophagus, and are taken up by the endodermal cells of the so-called "mesenteric filaments," where they are digested by a trypsin-like enzyme. In these animals digestion is entirely intracellular, though the ingesting cells are the parts of a specialized tissue. In other forms, still higher in the scale, although there is persistence of intracellular digestion, the extracellular process begins to be developed. Thus in certain mollusca the solid food is taken into the intestinal canal, where it first undergoes a preliminary digestion by secreted intestinal juices. After it has

[8] Mouton. *C. R. de l'Acad. des Sciences*, Vol. 133, 1901.

been reduced to small amorphous particles in this way, these are seized by the ameboid cells, and intracellular digestion completes the process which has been begun extracellularly.

As we study the process among higher animals, it appears that, among vertebrates, the intracellular methods of digestion have been, at least for normal metabolism, entirely displaced by the extracellular as it occurs in the intestine, where solid particles are rendered completely amorphous, dissolved, and reduced to a diffusible condition by the digestive juices before they are offered to the cells for utilization. However, the capacity for intracellular digestion is not entirely lost, and is retained of necessity in certain body cells. For were there not such an emergency arrangement the body would lack an available mechanism with which to meet such accidents as extravasations of blood, or the entrance of bacteria and other foreign solid particles into the tissues. It seems reasonable to classify both the phagocytic action of body cells and the formation of anti-bodies in the blood plasma, primarily as emergency devices for the digestion of foreign materials both formed and unformed which, under abnormal conditions of injury or disease, penetrate into the physiological interior of the body (blood stream or tissue spaces), and must be disposed of.

In the lowest animals the single cell is called upon to perform all necessary functions. In the course of evolution, however, as the body becomes more and more a community of many cells, a division of labor takes place which is expressed morphologically in the differentiation of tissues and organs, and physiologically in the adaptation of individual tissue cells to the performance of specialized functions. Nevertheless, it is necessary, both for certain normal processes, as well as for provision against such complex emergencies as those mentioned, that certain cells of the complex community should retain the primitive abilities of the more independent cells of the lower forms. Thus, among many animals, the phagocytic action of cells performs definite services in the course of normal development. This is seen most markedly in some insects (diptera) in which the destruction of larval organs, useless to the adult animal, may be entirely accomplished by the action of phagocytic cells, and a similar process may accompany the transformation of the tadpole to the adult in many amphibia.[9] In higher animals the removal of extravasations of blood is accompanied by a train of occurrences which is readily subjected to study.[10] In such cases the leukocytes rapidly enter the area of extravasation and an engulfment of the blood cells occurs, followed by a process of digestion entirely analogous to the digestion of similar blood elements by the various forms of intestinal hemamebæ. In the latter case it is a process of normal digestion, in the

[9] See Henneguy. "Les Insectes," Paris, 1904, p. 677.
[10] Langhans. *Virchow's Archiv,* Vol. 49, 1870.

former an emergency procedure carried out by virtue of the retained ancestral characteristics of the special phagocytic cells.

The leukocytes, whose chief functions seem to be associated with such processes of intracellular digestion, may, therefore, be looked upon as cells retaining primitive characteristics for definite physiological purposes. We shall see, however, that, to meet exceptional conditions, the process of phagocytosis may be carried out also by many other cells which are associated ordinarily with functions entirely apart from this phenomenon.

During normal life in higher animals, too, constant destruction of red blood cells by phagocytosis takes place in the spleen and liver, and is described by Dickson [11] as occurring in the bone marrow as well; and similar phagocytosis of red cells is seen in the hemolymph nodes. It is claimed by Metchnikoff, furthermore, that many of the degenerative and retrogressive processes which take place in the human body are carried on by the mechanism of phagocytosis. The rapid return of the puerperal uterus to the normal state is explained in this way, and work by Helme [12] seems to show that there is an actual phagocytosis of the hyperplastic uterine musculature during this period. The atrophic changes of senility, too, are attributed by Metchnikoff [13] to the same processes. The involution of the ovaries is accompanied by active phagocytosis of portions of this organ, and Metchnikoff claims further to have shown that the degeneration of the nervous system during old age is accomplished by the phagocytosis of nerve cells by phagocytic elements derived either from the leukocytes or the neuroglia, or from both.[14] The whitening of the hair, both in human beings and in old animals (dogs), is similarly due, he claims, to phagocytosis of the pigment by cells which wander in from the root sheaths. It is, up to the present time, impossible to determine the stimulus by which this phagocytosis is initiated.

Since the subject is a very important one, many studies have been made to determine which cells of the body of higher animals can take in and digest foreign particles and to classify them according to this power. Metchnikoff has distinguished between the "motile" and "fixed" phagocytes, the former the leukocytes of the circulating blood, the latter certain connective tissue cells, endothelial cells, splenic pulp cells, and certain cellular elements of the lymph nodes,

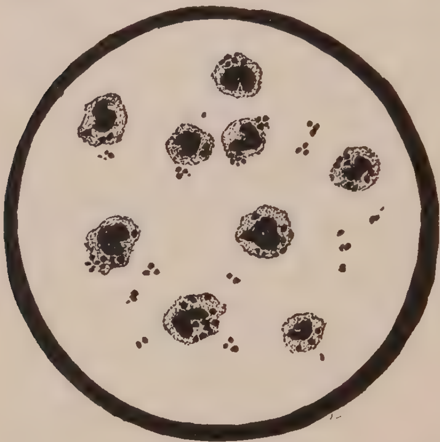
[11] Dickson. "The Bone Marrow," Longmans, Green, London, 1908.

[12] Helme. *Transact. Roy. Soc. of Edinburgh,* Vol. 35, 1889. Cited from Metchnikoff.

[13] Matschinsky. *Ann. de l'Inst. Past.*, Vol. 14, 1900; Vol. 15, 1901.

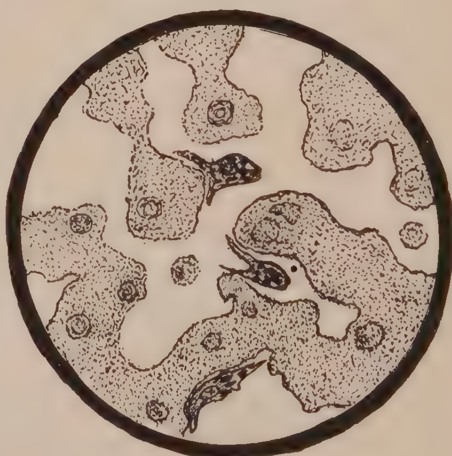
[14] That the leukocytes are concerned in the destruction and resorption of dead tissues has been shown by Leber especially (Leber, "Die Entstehung der Entzündung," Leipzig, Engelmann, 1891). An accumulation of leukocytes about a bacterial focus or from any other stimulus is followed by tissue lysis due to leukocytic enzymes.

the neuroglia tissue, and, in fact, all phagocytic cells which are ordinarily confined to some definite localization in the body. Among phagocytic cells Metchnikoff further distinguishes between "microphages," by which he designates the polymorphonuclear leukocytes of the circulating blood and "macrophages." The macrophages include the fixed cells mentioned above, together with the large mononuclear elements of the blood, in short, all phagocytic cells except the microphages.



POLYNUCLEAR LEUKOCYTES TAKING UP STAPHYLOCOCCI.

Although no absolute functional differentiation is possible between the two, it is true, in a general way, that the microphages are concerned primarily with the phagocytosis of bacteria and especially of those which invade acutely, while the macrophages are concerned especially with the resorption of cellular detritus, foreign bodies, and such bacteria as are more chronic in their activities, or are peculiarly insoluble. On the other hand, microphages may take up foreign particles and bacteria of all kinds under suitable conditions, and no sharp line can be drawn between the two varieties in this respect. Metchnikoff further believes that the two classes of phagocytic cells differ in the nature of the protective substances they secrete and furnish in the blood plasma. This, however, is a problem concerning which there is much difference of opinion and which calls for separate discussion in another place.



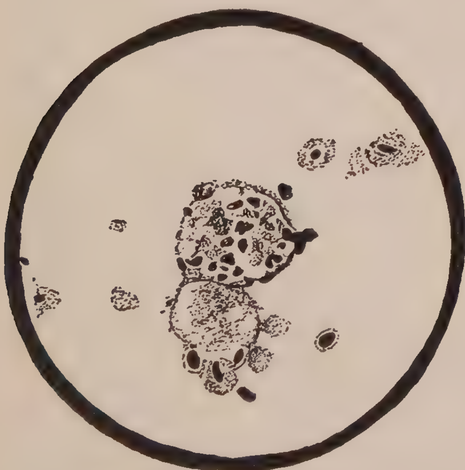
KUPPER CELLS CONTAINING MALARIAL PIGMENT. DIAGRAMMATICALLY DRAWN FROM A SECTION OF MALARIAL LIVER KINDLY FURNISHED BY DR. R. LAMBERT.

The property of phago-

cytosis is therefore an attribute of a considerable number of different varieties of cells. In the circulating blood the polynuclear leukocytes are the most actively motile and phagocytic elements. The eosinophile cells may also take up foreign particles and bacteria, as may also the large lymphocytes. The small lymphocytes and mast cells are either entirely inactive in this respect, or, at least, possess phagocytic powers under exceptional circumstances only. This does not mean, however, that these last-named cells may not accumulate at the point of invasion nor that they may not play an important part in the defence of the body. It is well-known, of course, that, in tuberculosis and a number of other conditions, the lymphocytes may form the majority of the cellular elements which accumulate at the site of the lesion. Among the fixed cells of the body it is probable that phagocytosis may be carried on by cells of many different origins, though the identification of cells in tissues is often a purely morphological problem, and therefore fraught with many possibilities of error. Probably the most active fixed tissue cells are the endothelial cells of the blood vessels and those which line the serous cavities, the

Rat Leprosy Bacilli Grouped in the Remains of Dead Spleen Cells Growing in Plasma.

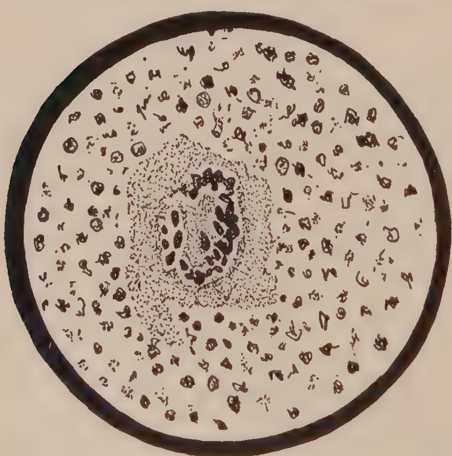
Drawn after illustration in Zinsser and Carey, *Journal of the A. M. A.*, Vol. 58, 1912.



Phagocytosis of Sensitized Pigeon Corpuscles by Alevolar Cells of Lung.

Drawing made after photomicrograph published by Briscoe, *Journal of Path. and Bact.*, Vol. 12, 1908.

sinuses of the lymphnodes, and of the spleen. However, there are many other cells in addition to these which may be phagocytic. The writer, with Carey,[15] has observed the active phagocytosis of leprosy bacilli by cells, probably of connective tissue origin, growing from plants of rat spleen in plasma. Phagocytosis by the cells lining the alveoli of the lungs has been observed by Briscoe.[16] This author made the interesting observation that in cases of mild infection such cells can free the lungs of micro-organisms entirely without aid from the leukocytes of the circulating blood. It is these cells, too, which, in the ordinary conditions of life, take up the inhaled particles of dust and are, therefore, often spoken of as dust cells. The origin of the dust cells has often been the subject of controversy. In the embryo the alveoli of the lung, like the bronchi, are lined with columnar cells which are transformed into flattened epithelium as the alveoli expand at the first inspirations after birth. These flattened cells, which constitute the alveolar or dust cells, are probably of epithelial origin, and as such are probably the only epithelial cells which act as phagocytes under ordinary conditions. Although no positive general statement is justified, we can yet say with reasonable accuracy that among the phagocytic fixed tissue cells the most important are the connective tissue and endothelial cells.



Giant Cell in Tuberculosis.

The type of phagocytosis and the variety of cell which participates in it seem to depend to a great extent upon the nature of the substance which incites the process, or rather at which the process is aimed. Thus the large cells which, in tissues, take up the leprosy bacillus, those which are characteristic of tuberculous foci, or those caused by blastomycetes, or by foreign bodies, all have special appearances which are sufficiently characteristic to have diagnostic value. However, it is difficult to determine with certainty the origin of the cells which participate. The chemical nature of the substances taken up, moreover, often complicates the phagocytic process in such a way that different cellular elements are enlisted in succession in order that the ingested substances may be disposed of. Thus tubercle

[15] Zinsser and Carey. *Jour. A. M. A.*, March, 1912, Vol. 58.
[16] Briscoe. *Jour. Path. and Bacter.*, Vol. 12, 1907.

or leprosy bacilli which are injected into an animal may be at first taken up by polynuclear leucocytes or microphages, by which they may even be carried into the lymph channels and distributed, perhaps to the detriment of the host. But these cells, probably because they lack a lipolytic ferment by means of which the waxes of the acid-fast organisms can be digested, cannot destroy the bacteria, which are then attacked by other cellular elements at the site of their final deposit.

That fixed tissue cells as a matter of fact play a very important rôle in the disposal of invading bacteria is becoming more and more clear. Kyes [17] showed a few years ago that the immunity of the pigeon to pneumococcus infection is largely due to active removal of the bacteria by the Kupfer cells in the liver. Bull's [18] observations on the intravascular agglutination of typhoid bacilli which are then phagocyted by cells in the liver and spleen point in the same direction, and recently Hopkins and Parker [19] in our laboratory have shown that streptococci injected into rabbits and cats are rapidly removed from the circulation, the removal being to a great extent due to phagocytosis carried on by the endothelial cells in the lungs and by similar cells in the liver and spleen.

In many such cases the further resolution of the foreign substance is accomplished by an important type of phagocytosis which is characterized by the formation of the so-called giant cells. These cells are of varying appearance in different conditions and locations. Thus the giant cells which form about foreign bodies, such as the small cotton fibers occasionally left in wounds, or injected particles of paraffin or iron splinters, etc., are quite characteristic and distinct from the giant cells of tuberculous foci, or of rhinoscleroma, glanders, or leprosy. They are all large cells, containing often numerous nuclei which form either by the fusion of several cells, as claimed by Borrell,[20] Hektoen,[21] and others, or by the cleavage of the nuclei alone, without coincident divisions of the cytoplasm.

Although it is, of course, impossible to decide definitely upon purely morphological grounds, the researches of Hektoen especially would lead one strongly to favor the former view. It is equally difficult to decide the origin of giant cells, and endothelial, connective tissue, and even leucocytic origin has been claimed for them. Yet in no case has it thus far been possible to actually observe their formation by a method which could positively decide this point.

In order to gain a clear conception of the participation of phagocytes in the response of the body to injury or invasion, it will be useful to follow out the process of inflammation as it occurs in the

[17] Kyes. *Jour. Inf. Dis.*, Vol. 18, 1916, p. 272.
[18] Bull. *Jour. Exp. Med.*, Vol. XX, p. 237.
[19] Hopkins and Parker. Paper in Manuscript.
[20] Borrell. *Ann. de l'Inst. Past.* 7, 1893.
[21] Hektoen. *Jour. Exp. Med.*, 3, 1898, p. 21.

higher animals. Inflammation may be incited by a large number of agencies—chemical irritants, mechanical injury, or even by the introduction of inactive and isotonic substances such as broth or salt solution.[22] Yet in these cases the response, though essentially similar in principle to that following invasion by bacteria, lacks certain features especially interesting in the present connection, and it will be most profitable for our purpose to consider in detail the result of infection with pathogenic micro-organisms.

If an emulsion of pyogenic staphylococci is injected into an animal subcutaneously the site of injection will soon become reddened and swollen and microscopic examination will show, within a few hours, a swelling and engorgement of the blood vessels.

The injected cocci will be found to lie partly scattered in the tissue spaces, in part within polynuclear leukocytes and connective tissue cells which have begun to ingest them. The tissue space will be swollen and stretched by the exudation of blood serum from the vessels. This condition will begin in from 4 to 6 hours after injection and increase during the next 24 hours in extent and severity, according to the quantity and virulence of the cocci injected. The conditions which precede the wandering of the polymorphonuclear leukocytes out of the vessels have been carefully studied in such thin tissues as the mesentery of a frog after injury by trauma or acid. Within the vessels of the affected area there is at first an acceleration of the blood stream, then a dilatation of the capillaries and a slowing of the current. Leukocytes may now be observed moving more slowly than the main stream, and keeping close to the periphery along the walls of the vessels. Here and there they seem interrupted in their movements and adhere to the vascular wall. A little later these cells appear to pass through the wall of the vessel by sending out pseudopodia which slowly penetrate it. Adami

Diagrammatic Representation of Leukocytes Wandering Through Capillary Walls.
Adapted from Ribbert, "Lehrbuch der Allgemeinen Pathologie," p. 337.

[22] Adami. "Inflammation," Macmillan, London, 1909.

states that if, at this stage, the tissues be excised, fixed in osmic acid, and stained, leukocytes may be seen crowding the inner surface of the vessel in all stages of transition from its anterior to the lymph spaces on the outside.

In the staphylococcus infection, after from 12 to 48 hours, there will be seen the results of an active and destructive struggle between the invading bacteria and the defending cells. In the center of the area of invasion tissue has been destroyed and disintegrated. Amid the necrotic detritus, closely packed, lie leukocytes and cocci and active phagocytosis has taken place. In some cases the intracellular bacteria appear swollen and disintegrating, in others the leukocyte itself, overcome by the larger number of bacteria it has taken in, becomes vacuolated, indefinite in outline, and apparently is being itself destroyed. The presence of blood serum, which is aiding in the destruction of bacteria both by its bactericidal powers and its reënforcement of the phagocytic process, renders this mass fluid or semi-fluid, and the whole mixture constitutes what is known as pus. Around the periphery cocci and leukocytes become more scattered and sparse, and bacteria, together with leukocytes, loaded with cocci. may be seen lying within large mononuclear cells (macrophages). Whether the process goes on to further extension or is eventually walled off into a distinct abscess by the formation of granulation tissue and new connective tissue depends upon the balance of forces between attacking agent and defensive factors.

If we inject a similar emulsion of cocci into the pleural or peritoneal cavity of an animal a process similar in principle may be observed.

Normally the peritoneum contains a small amount of this serous fluid and a moderate number of white blood cells, chiefly lymphocytes. When any substance, broth or salt solution, an aleuronat or a bacterial emulsion, is injected into the peritoneal cavity, there follows a brief period during which there is a diminution of the free cellular elements in the peritoneal fluid. At this time there is a clumping of cells in the folds of the omentum and mesentery, a transient stage of flight away from the point of injury. This, however, is soon over. Within one to two hours an active immigration of leukocytes into the serous cavity occurs and if, during the next 12 to 24 hours small quantities of fluid are, from time to time, withdrawn with a capillary pipette, a rapid and constant increase of leukocytic elements, chiefly of the microphage or polynuclear type, is observed. If the injected substance has been a sterile, harmless fluid, a gradual return to normal within 48 hours then ensues. If, however, we have injected bacteria, a struggle similar to the one described above takes place within the peritoneum, and active phagocytosis of the micro-organisms takes place.

Let us suppose that the injected bacteria have been small in

quantity and moderate in virulence. In such a case a rapid phagocytosis gradually rids the fluid of micro-organisms and within 24 hours after injection few, if any, free bacteria are visible.

A little exudate taken at this time shows large numbers of microphages varyingly crowded with well-preserved and disintegrating bacteria. Some of the phagocytes, having literally taken up more than they can digest, are vacuolated and disintegrating, but, in general, the victory lies with the cells. A little later large mononuclear elements appear, and here and there will be seen to take up dead leukocytes together with ingested cocci. In this way gradually a cleaning out of the peritoneum takes place, the animal recovers, and the peritoneum returns to normal.

Let us suppose, on the other hand, that the bacteria injected are in larger doses and of greater virulence. In such a case, after a period of active phagocytosis, there may be a gradual increase of bacteria over leukocytes. The phagocytic cells are found to be undergoing degeneration in larger numbers, the free bacteria increase, and the impending death of the animal can often be foretold by the appearance of the exudate. Finally, the peritoneal fluid may consist chiefly of free and rapidly multiplying bacteria with a practical absence of phagocytic cells.

In all of the processes so far as described the burden of the defence has fallen upon the microphages or polynuclear leukocytes, while the macrophages—endothelial and connective tissue cells—have taken a purely secondary part in the reaction, forming, to some extent, a second line of defence, or, more probably, taking part only in the final removal of degenerated and disintegrating combatants and tissue detritus. In order to obtain a complete conception of phagocytosis in its entire significance it will be necessary to consider a further example, namely, the process which takes place within tissues in the course of the efforts of macrophages to remove bacteria and other substances which, either because of their insolubility or for other unknown reasons, are refractory to the attacks of the microphages. Since we are interested in this subject chiefly from the point of view of the defence against bacteria, we may illustrate this process best by the description of the reaction which takes place when tubercle bacilli become localized anywhere within the animal body.

When tubercle bacilli are injected into the peritoneum they are actively taken up by the polynuclear leukocytes just as are other bacteria and many entirely inactive solid particles. A similar ingestion by microphages may take place in the folds of the intestinal mucosa if tubercle bacilli are fed to guinea pigs. However, this preliminary phagocytosis is probably of but secondary significance in the combat of the body against tuberculosis, since it has still to be shown that polynuclear leukocytes are capable of digesting and destroying acid-fast bacilli. Indeed, much evidence tends to show

that the ingestion of tubercle bacilli by microphages may be a detriment to the host, since the bacilli by this means are carried through the lymphatics and variously distributed throughout the body. Polynuclear leukocyte extracts, though containing, as we shall see, proteolytic enzymes, do not, according to Tschernorutzky, contain any lipase, and it may well be that for this reason they are unable to attack the waxy substances which form an integral part of these organisms. This is in keeping with the observations made by Terry in our laboratory, that rat leprosy bacilli may be kept within leukocytes for weeks without losing their acid-fast properties, whereas the same bacilli, as the writer and Cary found, were rapidly disintegrated in spleen cells growing in plasma. Moreover, it is well known that the estimation of tuberculo-opsonin contents of the sera of tuberculous patients has been peculiarly unsatisfactory in throwing light on the progress of the disease. It would seem, therefore, that in this disease, as well as in others caused by acid-fast organisms, the microphages play only an unimportant part in the defence of the body.

On the other hand, when tubercle bacilli are deposited either in a lymphnode (through the vehicle of leukocytes) or in a capillary anywhere by the blood stream, a train of cellular changes is initiated in which the predominant part is played by the macrophages. The tubercle bacilli so deposited are rapidly surrounded by large mononuclear cells, probably endothelial in origin. Some of the microorganisms may even be phagocyted and taken into these cells. These cells, spoken of as "epithelioid cells," surround the clump of bacteria in more or less concentric rings, and around these there is an accumulation of leukocytes, largely of the lymphocyte variety, with an admixture of a very few microphages. Then by the fusion of endothelial cells, or possibly by division of the nuclei of some of these cells within the individual cell bodies, giant cells are formed which take up the bacilli. The further progress of the tubercle now greatly depends upon the balance of power. Often such a tubercle may heal, possibly because of complete intracellular digestion of the bacilli. On the other hand growth and multiplication may lead to a slow and dry necrosis of the center of such a mass of cells, leading to the condition spoken of as caseation. Epithelioid cells lose their outlines and staining properties, and go to pieces. The center of the lesion is a grumous mass, the periphery shows a few giant cells and connective tissue proliferation.

It is always surprising to those who study these lesions for the first time how rarely they succeed in finding tubercle bacilli in microscopic sections prepared from such tubercles by the ordinary Ziehl-Neelsen method of staining. Repeated and careful examination of such material may fail to reveal any acid-fast organisms, though inoculation into guinea pigs is nevertheless successful, pro-

ducing typical tuberculosis. Much[23] has studied this peculiar state of affairs particularly and has shown that, although such lesions may show no tubercle bacilli by the Ziehl-Neelsen carbol-fuchsin method, staining by a modified Gram technique will reveal numerous Gram-positive rods and granules which have lost their acid-fast properties. This, too, if true, and the evidence is very much in its favor, would point to an ability of the macrophages to digest the waxy substance of the tubercle and other acid-fast bacilli, a property not possessed by the microphages. It may, of course, mean on the other hand that the tubercle bacilli in the lesion have not developed the waxy condition.

Chemotaxis and Leukocytosis

The part played by the phagocytic cell in the defence of the body against the entrance of bacteria and other foreign substances consists, then, of two functionally different phases. The first is an active motion of the cells toward the point attacked, and their accumulation about the noxious agent, the second consists in the act of ingestion itself.

The motion of the leukocytes toward the invading substances indicates a sensibility on the part of the cell to changes in its environment incited by the foreign agent, and since the stimuli most likely to reach the leukocytes and bring about this alteration in the direction of their movements are chemical in nature, the phenomenon is spoken of as "chemotaxis." This term was borrowed from Pfeffer,[24] who studied similar phenomena in connection with many freely motile plant cells, spermatozoa, and bacteria. Since the change of direction brought about in a moving cell by such influences may be such as either to attract or to repel, the term "positive chemotaxis" is used to designate the former and that of "negative chemotaxis" the latter.

The property of chemotaxis is of vital interest in the present connection, since, whatever may be our opinion regarding the relative values of phagocytosis and serum protection in immunity, the great importance of the phagocytic process cannot be questioned, and any agency which repels the approach of the phagocytes must be a detriment, while any factor which attracts them is, of necessity, a powerful means of defence. In the investigations upon the nature of infectious diseases attention has been concentrated upon the phenomenon of phagocytosis, and the relations governing the act of ingestion have been very thoroughly studied. The details of the chemotactic phenomenon, however, though of equal importance, are much more

[23] Much. "Beiträge zur Klinik der Tuberk.," Vol. 8, 1907, Hft. 1 and 4.
[24] Pfeffer. "Untersuch. a. d. Botan. Inst. Tübingen," Vols. 1 and 2, 1884 and 1888.

obscure. A large part of our sparse knowledge in this connection, moreover, has been gained by studies not related to infection.

The stimuli which determine the motion of cells are, of course, not necessarily chemical, and extensive studies have been made upon the effect of light waves in this connection. Although these investigations are of great biological importance, they have little direct bearing upon the problems of tropism as related to bacteria and leukocytes and cannot therefore be considered here.

Some of the earlier researches upon chemotaxis were those made by Stahl [25] upon the slime-molds or myxomycetes. These organisms possess the power of ameboid motion, and were observed by Stahl to move toward or away from any given region, according to the nature of the substances with which they came in contact. Pfeffer subjected this phenomenon to closer analysis. Working with the spermatozoa of ferns, swarm spores, bacteria and infusoria, he elaborated an ingenious technique by means of which he was enabled to determine directly the negative or positive chemotactic properties of various substances in solution upon these motile forms. His technique was exceedingly simple. Capillary glass tubes, about 8 to 10 mm. long and 0.1 mm. in diameter, were sealed at one end in the flame, and then dropped into a watch-glass. The solution which was to be tested was poured over the tubes and the watch-glass then placed under the bell of an air-pump. When the air was evacuated and pressure reduced the tubes became partly filled up with the liquid. They were then removed, washed in water, and placed under a cover slip under which a preparation of the motile cells was swimming. Positive chemotaxis was indicated by entrance of the cells into the tubes, negative, by their refusal to enter. Failure of the solution to exert any chemotactic influence resulted in their moving into and out of the tubes indiscriminately.[26]

By this technique a large number of interesting observations were made which threw much light upon the causes underlying the movements of plant cells. For instance, in investigating the spermatozoa of the ferns it was found that they were attracted strongly by malic acid and its salts, while no other substance investigated approached these compounds in the intensity of positively chemotactic stimulation. From this Pfeffer concludes that the bursting of the fern archegonia is accompanied by the liberation of malic acid, this attracting the male to the female cell.

Similar experiments have been carried out since then by numerous naturalists, among them Buller,[27] Lidforss,[28] and Jennings,[29]

[25] Stahl. *Botanische Zeit.*, 1884.
[26] Buller. *Annals of Botany,* Vol. 16, No. 56, 1900.
[27] Buller. *Loc. cit.*
[28] Lidforss. "Jahrbücher f. wissensch. Botanik," 41, 1904.
[29] Jennings. "Behavior of Lower Organisms," Columbia Univ. Press, Macmillan, 1906.

and it has been found that in addition to malic acid compounds many other substances, organic and inorganic, occurring in plant cells and cell-sap exert positive chemotactic power. Lidforss has shown, for instance, that calcium chlorid in 0.1 per cent. solution may strongly attract plant spermatozoids (equisetum—horsetail). When the solution is concentrated to 1 per cent., attraction is still exerted, but the spermatozoids immediately lose their motility upon entrance into the fluid.

The same worker has shown that a substance which is positively chemotactic for one variety of plant cell may be negatively chemotactic for another, showing a certain selective variation which should be of great biological importance. Thus capillaries with a 1 per cent. solution of potassium malate actively attracted the spermatozoids of marchantia (a liverwort), while not a single spermatozoid of equisetum would enter these tubes. Löw [30] has applied these methods of study to the investigation of the chemotaxis of mammalian spermatozoa and found that these cells were actively attracted by weakly alkaline solutions.

Studies upon the factors determining the movement of bacteria and amebæ toward some substances and away from others have been numerous, and are valuable for the understanding of leukocytic chemotaxis, because they have led to the formulation of a number of important general theories. The fact that the motions of bacteria in suspensions are, to a certain extent, determined by the negative electrical charge which they all carry in neutral media, has been touched upon in the section on agglutination. Attempts on the part of Young and the writer to determine whether the attraction of leukocytes toward bacteria might be due to the carrying of an electropositive charge by the white cells have met with no result, owing so far to the failure to elaborate a reliable technique. However, this thought is not an impossible one and should be borne in mind.

That certain bacteria will wander actively toward a source of oxygen was shown by Engelmann's [31] classical experiment in which a diatom, half in the shade and half in the light, was surrounded by an emulsion of bacteria, and these were seen to collect about the lighted half only, where oxygen was being liberated by virtue of the chlorophyll. The extreme delicacy of chemotactic reactions is illustrated in these experiments in that Engelmann calculated that the bacteria reacted to one one-hundred billionth of a milligram of oxygen. The selective reaction of bacteria to various chemical substances, furthermore, has been shown by allowing different solutions to diffuse into bacterial emulsions from capillary tubes, and by observing attraction or repulsion from the point of contact.

The chemotaxis of leukocytes has opposed more difficulties to

[30] Löw. *Sitzungs Berichte kais. Akad. d. Wiss.*, Wien, Vol. 3, Abf. 3.
[31] Engelmann. *Arch. f. d. ges. Physiol.*, Vol. 57, p. 375.

direct study, since the conditions within the living body are subject to a large number of modifying factors, and experiments upon the isolated cells, in vitro, even under conditions of the most careful technique, are fraught with much unavoidable injury to the cells. However, enough has been learned to indicate that these cells are subject to the phenomena of chemotaxis or tropism just as are independent unicellular forms, and that they may be attracted or repelled by a variety of organic and inorganic substances. Leber [32] was one of the first to study this in his work upon inflammation. He found that leukocytes were actively attracted by powdered copper and mercury compounds, but not by powdered gold or iron. He also observed that dead bacteria exerted a similar positive chemotactic influence, and Buchner [33] later succeeded in extracting substances from various bacteria which possessed similar properties. It appears, from these and other investigations, that the power of stimulating positive chemotaxis is a general property of bacterial proteins, equally evident in bacterial extracts, dead bacteria, or the living organisms. It is likely, therefore, that the attraction of leukocytes toward the point of bacterial invasion is, in part at least, due to the properties of the bacterial proteins themselves. That this, however, is not the whole story is evident from the work of Massart and Bordet,[34] who showed that the products of cell destruction and disintegration possess similar positively chemotactic properties. This is true not only of the products of disintegrated tissue cells, but of those of the destroyed leukocytes themselves. Thus it appears that when any injury of tissue takes place, a stimulus which attracts leukocytes results, even when the injury is not accompanied by bacterial invasion. This would explain the participation of leukocytes in reactions to injury, and in inflammations not of bacterial origin, and their local accumulation following the injection of insoluble inorganic substances.

When bacteria are actually present, however, the added stimulus due to the diffusion of bacterial proteins probably increases the process to a degree often sufficient to meet the added requirements for protection. Following this, both the destruction of tissues, of bacteria, and of leukocytes may together exert a cumulative chemotactic power which continues the process proportionately with the extent of the lesion.

It is of the utmost importance, therefore, to ascertain whether or not any substances derived from bacteria may, under any circumstances, exert a repellent or negatively chemotactic power. If we infect an animal intraperitoneally with virulent bacteria, in doses

[32] Leber. *Fortschr. der Med.*, 1888; also "Die Entstehung der Entzündung," Engelmann, Leipzig, 1891.

[33] Buchner. *Berl. klin. Woch.*, Vol. 27, No. 30, 1890.

[34] Massart and Bordet. *Ann. de l'Inst. Past.*, Vol. 5, 1891.

sufficient to lead to death, and examine the peritoneal exudate just before the lethal outcome, we may observe that leukocytes are gradually disappearing, and that finally but a few will be present and the fluid will be swimming with free micro-organisms. In the same way it is well known that the diminution of leukocytes in the circulating blood—or even the failure of these cells to increase in the circulation in the course of such diseases as pneumonia, or general infections with staphylococci or streptococci—is seriously prognostic of fatal outcome. The conditions here observed point strongly to the existence of substances of negative chemotactic influence which protect the bacteria, not from phagocytosis itself, but from that necessary forerunner of phagocytosis, the approach of the leukocyte. It is necessary to draw this distinction since these phenomena are not merely, as often believed, "antiopsonic," but in truth largely "antichemotactic." It is true that Kanthack,[35] and more especially Werigo,[36] have denied the existence of negatively chemotactic bacterial products, the latter basing his assertion upon the observation that active phagocytosis occurs in the lungs, liver, and spleen of animals dying of infection with virulent germs. However, the arguments of these authors are not conclusive and a mass of experimental and clinical evidence which points to a direct failure of leukocyte accumulation in the presence of virulent bacteria in the animal body would alone suffice to render such conclusions unlikely. Moreover, strong evidence in favor of the existence of negatively chemotactic influences is brought by the extensive experiments of Bail upon the so-called aggressins, discussed in another place, and such observations as those of Vaillard and Vincent[37] and Vaillard and Rouget,[38] which showed that the injection of a little tetanus toxin together with tetanus spores would prevent the ingestion of the spores by leukocytes, and thereby furnish an opportunity for germination and consequent fatal toxemia.

Similar observations have been made by Bésson[39] in the case of the bacillus of malignant edema by the use of the original technique of Pfeiffer. Capillary tubes containing the toxin remained free of leukocytes after subcutaneous introduction into guinea pigs, while similar tubes containing the culture medium alone, or the bacilli and their spores, attracted leukocytes in considerable numbers.

It is possible, of course, to interpret such phenomena as due to a failure of positive chemotaxis rather than to an active negative chemotaxis.

Although the phenomena of chemotaxis are most easily studied

[35] Kanthack. Quoted from Adami, *loc. cit.*
[36] Werigo. *Ann. de l'Inst. Past.*, Vol. 8, 1894.
[37] Vaillard and Vincent. *Ann. de l'Inst. Past.*, Vol. 5, 1891.
[38] Vaillard and Rouget. *Ann. de l'Inst. Past.*, Vol. 6, 1892.
[39] Bésson. *Ann. de l'Inst. Past.*, Vol. 9, 1895.

in extravascular inflammatory changes, there is none the less a regular and apparently purposeful attraction or repulsion of leukocytes evident in the circulating blood during infectious diseases. That infection of the body with many micro-organisms results in the increase of leukocytes, and that in others there is either no increase or even a decrease, is too well known and too generally applied in diagnosis and prognosis to warrant our giving up much space to a review of the facts. Nevertheless, the causes which lead to a leukocytosis in the one case, a leukopenia in the other, are still very obscure and deserve discussion.

In the first place it is by no means certain whether a leukocytosis signifies an active discharge of new leukocytes from the bone marrow or whether it means simply an altered distribution in that the phagocytes accumulated in the lymphatic and other organs are attracted by chemotaxis into the peripheral circulation. Studies of the bone marrow during infection as well as the occasional appearance of myelocytes and other cells ordinarily found only in the bone marrow during health would point toward a participation of active bone-marrow hyperplasia in the increase of peripheral leukocytes. There is no good reason to doubt, moreover, that a chemotactic stimulus exercised in the circulation should withdraw leukocytes from any place of accumulation to the circulation. Probably both processes take part. When bacteria are injected into the circulation of an animal there is, at first, a moderate diminution of the leukocytes just as there is after injection of bacteria or other substances into the peritoneum. This is soon followed in most cases by a rapid and progressive increase, in which, whenever the leukocytosis is one of considerable degree, the polynuclear leukocytes preponderate. The extensive clinical study of the white cells in infectious disease of the human being give us more material for reasoning in this respect than we have available from animal experiment. Infection with invasive bacteria such as the pneumococcus (and Neufeld and others have shown that most lobar pneumonias are accompanied by pneumococcus bacteriemia), streptococci, staphylococci, and others is always accompanied by an increase of the leukocytes, while, in typhoid fever, influenzal infection, tuberculosis, and a number of other infections, the leukocytes do not increase and may even decrease. How are we going to account for this? That all these bacteria contain a substance which is positive in its chemotactic effects is easily demonstrated by injecting them into the peritoneum and observing an accumulation of leukocytes and a consequent phagocytosis, even in the cases of those organisms which do not call forth a leukocytosis in the blood of the diseased human being. Thus it has been our experience as well as that of others invariably to observe the rapid and complete polynuclear phagocytosis of both leprosy bacilli and tubercle bacilli after injection of these micro-

organisms into the peritoneal cavities of guinea pigs. Yet a chronic tuberculous peritonitis or pleurisy is characterized usually by an exudate which contains but few polynuclears and relatively many lymphocytes. A final explanation of these conditions is not possible at present. No adequate explanation for the selective accumulation of lymphocytes and the absence of polynuclear cells about tuberculous foci has yet been advanced. The absence of polynuclear leukocytosis may possibly be due to the great insolubility of these bacilli, in consequence of which little or no leukocytosis-stimulating substances are liberated.

Pearce [40] has suggested a similar reason for the absence of polynuclear accumulations about chronic localized lesions of any kind in which tissue encapsulation may prevent the contact of the inciting agents with the body fluids and there is a consequently slow or slight production of such chemotactic stimulating materials.

In typhoid fever, where the slight primary leukocytosis is rapidly succeeded by a leukopenia with a relative lymphocytosis, the conditions are somewhat different. Here, as in some other infections, as Friedberger and others have shown, we are dealing with a generalized infection by an organism which is easily subject to the action of alexin with consequent production of anaphylatoxin. (See chapter on Anaphylaxis.) This poison, it seems, exerts a negative chemotaxis, and probably during the height of the disease, therefore, leads to the low leukocyte count observed. That this is at least likely seems to follow from the studies which have been made upon the nature of the typhoid poisons, and also from the observation of Gay and Claypole, that typhoid-immune rabbits react to the infection of typhoid bacilli with a rapid and powerful increase in the polynuclear leukocytes, whereas similar injections into the normal animal lead to leukopenia.

If the supposition regarding tuberculosis, made above, is correct, it would follow that a sudden and considerable increase in the polynuclear leukocytes in a case of tuberculosis would indicate a discharge of organisms into the circulation and a tendency toward generalization of the infection in this manner. (See Weigert's view of the manner in which tuberculosis may spread by the destruction of the wall of a vein by a localized lesion.) However, although speculation in the absence of experimental proof is justified, it must not be forgotten that the problems of selective chemotaxis are too obscure to permit of our laying much weight on any of these views.

Gabritchewsky,[41] who investigated this subject extensively, has classified various substances according to their positively, negatively, or neutral chemotactic activities. It is not necessary to recapitulate these, but it is interesting to note that he found that some substances

[40] Pearce. *Jour. A. M. A.*, Vol. 61, 1913.
[41] Gabritchewsky. *Ann. de l'Inst. Past.*, Vol. 4, 1890.

which were positively chemotactic in certain concentrations became neutral or even negative when the concentration was altered.

We have seen that the action of the leukocytes in moving toward some substances and away from others is entirely analogous to similar phenomena occurring among lower, unicellular forms of life, and the explanations applied to the apparently conscious acts of the ameba, such as the motion toward and the engulfment of food, have been applied to the activities of the leukocytes as well. Many of the theories developed concerning the free living forms, however, have been easily excluded in the case of the leukocytes, because of the environment in which their activities are developed. Thus the many interesting reactions of paramecia and other organisms to light (heliotropism) have little bearing upon this subject, and the views based on the theories of orientation may be excluded on the ground of the symmetry of the normal leukocyte. The observations of Garrey,[42] that indicate that it is the dissociated ions of various acids and bases which are responsible for the directive stimuli exerted upon certain flagellates, may yet result in throwing some light upon leukocytic movements, especially if we can come to accept the conceptions of ion-proteins upheld by Loeb [43] and his pupils. However, the facts concerning these phenomena, as well as the possibility, previously mentioned, of the opposite electrical charges carried by the leukocytes and the substances attracting them cannot be regarded at present as more than interesting thoughts. Of more than merely speculative interest, however, are the views of chemotaxis which are based upon the study of conditions of surface tension. In order to consider these properly it will be useful to review briefly the fundamental principles governing these conditions.

The molecules of any fluid are held together by mutual attraction due to the force generally spoken of as cohesion. This force is exerted by like molecules upon each other in solids more strongly than in liquids, and in gases less strongly. Since we are dealing in this connection with occurrences taking place in liquids, we will restrict our consideration to these. The force of cohesion is influenced in a number of ways. Thus, for instance, heat reduces it, and this is the cause that solids are converted into liquids and liquids into gases, provided of course that the heat brings about no chemical change. In large masses of fluids the force of gravitation overcomes that of cohesion and larger masses of liquid assume the shape of the containing vessel. In smaller masses the force of cohesion tends to bring about the spherical shape. This comes about in the following way: Within the interior of a drop of liquid all the molecules attract each other, and since the force of attraction is equal in all directions it neutralizes itself, and the molecules are

[42] Garrey. *Am. Jour. Phys.*, 3, 1900.
[43] Loeb. *Am. Jour. Phys.*, 3, 1900.

uninfluenced by it, mobile and free. The molecules on the surface are in a different condition, however. They are subjected to the force of cohesion from within, but not from without, and are therefore drawn strongly toward the center. The result is the same as though the drop were subjected to pressure from without and the surface layers were in a state of compression. There is in consequence a constant tendency of all the surface molecules to be drawn toward the center and a resulting tendency to a diminution of the surface area. It is as though the surface of such a drop were a thin, elastic membrane which tended to contract and diminish in size and surface. The force with which this takes place is spoken of as surface tension,[44] and the energy underlying it is called, by Ostwald, surface energy. Since a drop of one fluid suspended in another with which it cannot mix is relieved of the disturbing factor of gravitation, its surface tension has the effect of contracting the small mass into a form which, for the given volume, will expose the smallest possible surface, and this is, of course, the sphere. It is for this reason that, if we shake up such systems as water and chloroform, or oil and water, the chloroform or the oil will be distributed through the water as small droplets. The degree of surface tension of any fluid is measurable by a number of reasonably accurate methods which may be found in any text-book of physics and which we need not consider here. It is of course dependent in each case upon the nature of the surrounding medium. We have taken into consideration above only the force which is exerted within the drop by the cohesion, that is, the attraction toward the center. This would be uninfluenced from without only in a vacuum. In nature the surface molecules, though forcibly drawn toward the center, are also affected from without by the attraction exerted by the molecules of the substances surrounding the drop. There is a constant balance, therefore, at any part of the surface of a drop of fluid between the cohesion tension from within and attractions from without. The resultant of the two forces determines the surface tension, which will be greater or less in inverse ratio to the attraction from without for any given drop, and a variation of the external attraction at different points on the periphery of the drop will naturally influence the shape of the drop. For a relief of attraction at one point would tend to permit that part of the surface to retract, and an increase in this attraction would tend to allow it to bulge, with the formation of a sort of pseudopod.

In studying the importance of surface tension [45] in determining the motions of unicellular organisms a number of important attempts have been made to imitate cell motion by means of the suspension of various substances of strong cohesive properties in liquid media. The

[44] Michaelis. "Dynamik der Oberflachen," Steinkopf, Dresden, 1909.

[45] For a thorough discussion of this phenomenon see also Gideon Wells, "Chemical Pathology," Saunders, 1911.

idea was suggested by Quincke,[46] and later by Bütschli,[47] but has been most extensively studied by Rhumbler.[48] The result has been the production of a number of "artificial amebæ" which in almost all respects behave like the living organisms. Thus if a small mass of mercury is placed into a dish filled with water acidified with nitric acid, and a small crystal of dichromate of potassium is dropped near the mercury, the dichromate will dissolve and a yellow cloud will gradually diffuse from it toward the mercury. As soon as the yellow cloud touches this it will begin to show change of form and to elongate in the direction of the dichromate, often moving toward it. The motion of the quicksilver will resemble with considerable accuracy that of an ameba moving toward a particle of food or sending out pseudopodia. A more striking and complete imitation is that obtained by Rhumbler when he placed a drop of clove oil into a mixture of alcohol and glycerin. The changes of surface tension produced upon the surface of the clove oil by the alcohol give rise to movements in the oil entirely analogous to those of motile cells in favorable media. The similarity has been extended even to the processes of engulfment of the food as observed among amebæ. Thus a drop of chloroform in water will flow about a particle of shellac and dissolve it. If a piece of glass coated with shellac is placed in contact with the drop it will engulf it, but will cast out the glass after the shellac coating has been dissolved away.

The similarity between phenomena purely referable to surface tension and those taking place in the living cells is therefore very striking and has been clearly analyzed in regard to its bearing upon leukocytic chemotaxis by Gideon Wells in his "Chemical Pathology." The chemotactic substances, diffusing to the leukocyte, will lower its surface tension on the side at which they come in contact. Pseudopodia will be thrown out on this side in consequence, and the leukocyte will move in this direction. The motion will be continued in this direction as long as the concentration of the chemotactic substance, and therefore the diminution of surface tension is greater on this side than on other parts of the periphery, until a point is reached at which the chemotactic substance is equally diffused on all sides, and motion will cease. The actual engulfment may then occur or the nature and concentration of the chemotactic substance may be so great that injury is done to the leukocyte. Whether or not the purely physical explanation of chemotaxis tells the whole story it is of course not possible to decide. At any rate, it furnishes a rational basis for

[46] Quincke. Quoted from H. G. Wells, "Chemical Pathology," Saunders, 1907.

[47] Bütschli. "Untersuch. über mikroskopische Schaume und das Protoplasma." Leipzig, 1892. See also H. G. Wells, *loc. cit.*, pp. 220 *et seq.*

[48] Rhumbler. *Arch. f. Entwickelungs Mechanik,* 1898.

the study of the phenomenon more promising than any of the others so far offered.

It is true, on the other hand, that such a theory in no way accounts for the apparently selective positive chemotaxis which is exerted by different substances. Thus the preponderance of polynuclear leukocytes in foci and serous cavities containing organisms like staphylococci, meningococci, streptococci, and others is in contrast to the lymphocytic accumulation in the pleural, subarachnoid, and peritoneal spaces infected with tubercle bacilli. Some writers have spoken, therefore, of active and passive leukocytosis according to whether or not the cells attracted seemed to possess ameboid motility. That surface tension phenomena alone do not account for this is clear. But it must be remembered that even tubercle bacilli, though eventually attracting few polynuclears and many lymphocytes, will cause an active polynuclear accumulation in the peritoneum and pleura when first injected, and are actively phagocyted. Later when the lesion is established and the bacilli are lodged in the tissues the polynuclears give way to the lymphocytes, which even then never accumulate in such proportion as do the microphages in acute suppurative lesions. It may well be that the chemotaxis originally attracting the polymorphonuclear leukocytes is the same in every case, but that a continued irritant, especially one well surrounded by tissue elements as are the organisms within the tubercles, may cease to exact any chemotactic influence, the accumulation of inactive lymphocytes possibly being due to a progressive death of these cells carried into the neighborhood of the lesion by the normal circulation of the lymph.

CHAPTER XIV

THE RELATION OF THE LEUKOCYTES AND OF PHAGOCYTOSIS TO IMMUNITY

In Metchnikoff's earliest work upon the daphnia or water flea he observed clearly that there was a direct relation between the degree of phagocytosis and the outcome of the infection. When phagocytosis of the invading yeasts was energetic and complete the daphnia recovered. When the yeast cells penetrated the intestinal wall of the daphnia in large numbers, and were enabled to multiply before the phagocytic cells could accumulate in sufficient numbers to engulf them, then the body of the daphnia was soon swamped with the parasites and death ensued.

This simple observation fostered the thought that the basic principle underlying all processes of immunity was represented in this struggle between the invading bacteria and the phagocytic cells. To the activity of the latter, entirely, he attributed the power of resistance.

In support of this contention Metchnikoff and his pupils have marshaled many facts, most of which are set forth in his classical work "L'Immunité dans les Maladies Infectieuses." It will be manifestly impossible here to do more than outline the plan of study which these investigations have followed and the conclusions to which they gave just foundation.

The original study upon the infectious disease of daphnia led to analogous experiments upon higher animals and, by the prolonged and patient investigations of Metchnikoff and his pupils, it was shown that, throughout the field of infectious disease, there was a striking parallelism between the resistance of the infected subject and the degree of phagocytosis which occurred.

Earlier studies concern themselves chiefly with the natural immunity possessed by many animals against certain infection. The infectious disease which at this time had been most thoroughly studied was anthrax, and Koch had shown that frogs and other cold-blooded animals were markedly resistant against this micro-organism. Taking advantage of this observation, Metchnikoff studied the phagocytosis of anthrax bacilli in frogs and found that it took place rapidly and effectively, all of the injected bacilli being soon engulfed by the accumulating cells. Similarly, active phagocytosis of anthrax bacilli was demonstrated in such naturally resistant animals as dogs

and chickens, while almost no cell ingestion occurred in delicately susceptible animals like guinea pigs and rabbits. Rats, on the other hand, more resistant to anthrax than guinea pigs, less so than dogs, showed a degree of phagocytosis intermediate between that observed in the cases of the other animals mentioned above. And yet, in these more susceptible animals, the normal bactericidal action of the blood upon anthrax bacilli, though never extreme, was often more marked than that of the naturally immune animals mentioned above.

It is well known, for instance, that the serum of dogs possesses almost no bactericidal properties for anthrax bacilli,[1] although the animals are highly resistant to this infection, while the serum of rabbits is probably more strongly bactericidal for these bacilli than the serum of most other animals, and yet rabbits are extremely susceptible. That the lack of bactericidal powers of the serum is not always a sign of susceptibility on the part of the animal was shown as early as 1889 by Lubarsch. (We must remember, however, that lack of bactericidal power does not necessarily mean lack of sensitizer. For bacteria may be sensitized without being killed extracellularly as can be shown by the alexin-fixation reaction.)

The study of anthrax infections was a peculiarly fortunate choice of subject, since in this bacillus resistance to serum lysis is especially well marked and phagocytosis seems indeed to be the chief mode of bacterial destruction. Studies analogous to those originally made with anthrax, however, were subsequently carried on with streptococci, pneumococci, and staphylococci chiefly by Bordet,[2] Marchand,[3] and others, and results coinciding with those of Metchnikoff were obtained. In every case naturally resistant animals showed marked phagocytosis, and susceptible ones failed to show it to the same degree. It is a strong support of the same opinions, too, that Marchand's studies, later extensively confirmed, showed that the more virulent and invading strains of streptococci, the less active is the phagocytosis—a converse, but equally conclusive, observation.

Further support for this point of view is manifold and cannot be considered with anything like completeness. We may refer briefly, however, to the experiments of Vaillard, Vincent, and Rouget [4] with tetanus, and those of Leclainche and Vallée [5] with symptomatic anthrax, because they are especially valuable in illustrating the importance of phagocytosis in another class of infection. The poisons of these micro-organisms are extremely toxic for rabbits, and if a small amount of culture material, together with agar, broth, or any foreign substance which may inhibit or divert phagocytosis from

[1] Petterson. *Centralbl. f. Bakt.*, 1, 39, 1905.
[2] Bordet. *Ann. de l'Inst. Past.*, Vol. 11, 1897.
[3] Marchand. *Archiv. de med. Exp.*, Vol. 10, 1898.
[4] Vaillard, Vincent, and Rouget. *Ann. de l'Inst. Past.*, Vols. 5, 6, 1891-1892.
[5] Leclainche and Vallée. *Ann. de l'Inst. Past.*, Vol. 14, 1900.

the spores, is injected into these animals rapid proliferation and death with toxemia result. If, on the other hand, the spores are carefully washed of foreign material and toxin rapid phagocytosis results and the animals recover.

The parallelism which was followed out so extensively between natural immunity and phagocytosis was even more closely marked in the case of artificial acquired immunity. The first observations of this kind made by Metchnikoff, again on the subject of anthrax infection, were carried out by the active immunization of rabbits. The subcutaneous injection of virulent anthrax bacilli into normal rabbits is usually followed by a rapid growth of the bacteria, with much serous exudation but hardly any leukocytic accumulation. In immunized animals, on the other hand, the bacilli are taken up by hosts of phagocytes, just as this occurs in naturally resistant dogs or other animals. Similarly Bordet[6] has shown that cholera spirilla injected into the blood stream of cholera-immune animals are taken up by leukocytes even before they can be subjected to lysis by the circulating lytic antibodies.

It would add little to clearness were we to multiply the examples in which it has been demonstrated that the acquisition of increased resistance is accompanied by enhancement of the phagocytic process. This statement may be regarded as an axiom, and indeed our later discussions of the *opsonins* and *bacteriotropins* will show clearly why such a state of affairs is to be expected. Taken by itself, however, it does not necessarily prove that the destruction of the invading germs is entirely due to the leukocytes. It might still be possible that the bacteria are injured or even killed by the antibacterial serum constituents before they can be taken up and carried away by the cellular elements; the phagocytes then would act only as scavengers for the removal of the dead bodies. Indeed, this opinion was long held by a number of the adherents of the purely humoral school. However, such a point of view is no longer tenable—especially in the light of the later opsonin studies just referred to. Moreover, long before these more recent studies it was clear that bacteria may often grow within the leukocytes—finally destroying these—and that they may even remain fully virulent after ingestion. For, as Metchnikoff showed, if guinea pigs were injected with a little of the exudate formed after the injection of anthrax bacilli into immunized rabbits (an exudate in which there were no longer any extracellular bacteria because of energetic phagocytosis) death often resulted. It was clear, therefore, not only that the ingested bacteria were still alive, but that they were, at least in part, still fully virulent.

A further method of investigation employed by Metchnikoff in his endeavors to prove his point consisted in the attempt to demon-

[6] Bordet. *Ann. de l'Inst. Past.*, Vol. 9, 1895.

strate that virulent bacteria could be protected from destruction in the bodies of resistant animals if the leukocytes could be held at bay. This resulted in a number of ingenious experiments, the most convincing of which is the one carried out with anthrax bacilli and frogs by Trapetznikoff.[7] Anthrax spores were inclosed in little sacks of filter paper and these were introduced subcutaneously into frogs. In consequence the spores, bathed in the tissue fluids, but protected from phagocytosis, developed into the vegetative forms, multiplied, and remained virulent for several days. Taken up by the frog's phagocytes under ordinary conditions, they would rapidly have been taken up, digested, and destroyed. Here again it was shown that the body of fluids alone were unable to dispose of the bacteria and that the natural resistance of the frog was due entirely to phagocytic processes.

Other experiments have been aimed at a general reduction of phagocytic activity by the use of narcotics. Thus, Cantacuzene[8] showed that animals treated with opium are very much more susceptible to infection than are normal controls. And since opium markedly inhibits the activity of the white cells it may possibly be that these experiments furnish a further support for Metchnikoff's opinion. At any rate, it is worth noting that, even though these experiments are not convincing in their assertion that the increased susceptibility was due entirely to the interference with the leukocytes, they indicate very definitely the inadvisability of using morphin and similar narcotics in infectious diseases.

It is quite clear at any rate, then, that the process of phagocytosis increases in energy as immunity is acquired and, so far, Metchnikoff's assertions are entirely upheld by later knowledge. In his contention that *all* properties upon which the resistance of the animal against infection depends center directly or indirectly in the phagocyte, however, many subsequent amendments have been necessary, which will become self-evident in the following discussions of individual phases of the destruction of invading bacteria.

We cannot at the present time attempt to correlate these extreme views of Metchnikoff with the equally extreme opinions of those investigators who formerly attributed immunity entirely to the properties of the body fluids, assigning to the cellular activities a merely secondary rôle. Many of the apparently opposed contentions have become reconciled, and we now realize that neither process alone tells the whole story, both being parts of the complicated correlated processes which together constitute the mechanism of resistance. It was indeed to the eager controversy between the two schools that we owe much of the clearness of conception which recent years have given.

After the bacteria are taken up by phagocytes they undergo a

[7] Trapetznikoff. *Ann. de l'Inst. Past.*, Vol. 5, 1891.
[8] Cantacuzene. *Ann. de l'Inst. Past.*, Vol. 12, 1898.

gradual disintegration or dissolution comparable to that by which a particle of food is digested within the cell body of a rhizopod. With the exception of such particularly insoluble micro-organisms as the tubercle bacillus, the leprosy bacillus, blastomyces, and a few others, there is in all cases an eventual complete resolution of the bacterial body. As in amebæ the digestion takes place often after the formation of digestive vacuoles, and by staining at this time with neutral red it may be demonstrated that the process goes on in a weakly acid environment.

Problem of the Leukocytic Origin of Alexin.—Metchnikoff naturally assumed, therefore, that the intracellular digestion of bacteria by microphages (polynuclear leukocytes), or of cellular elements, etc., by macrophages, was a process carried on most probably by enzymes, and that these enzymes were identical with the bactericidal bodies described as "alexin" and "sensitizer" or "amboceptor" in the blood stream. To follow without confusion the development of his ideas, however, it is necessary to bear in mind that much of his earlier work was done at a time when the discovery of the coöperation of two substances in bacteriolysis and hemolysis (the amboceptor and the complement) had not yet been made by Bordet, and when the bactericidal effect of normal serum was attributed entirely to a single substance—the alexin of Buchner.

Buchner [9] himself had suggested that alexin was an enzyme-like body probably derived from the leukocytes.

In his experiments Buchner had noticed that escudates, produced by intrapleural injections of aleuronat in rabbits and dogs, possessed a bactericidal value for *Bacillus coli* which exceeded the bactericidal power of the blood serum itself. The influence of active phagocytosis could be excluded by the fact that the leukocytes of the exudate had been killed by repeated freezing and thawing. Similar results were obtained by Hahn [10] with *B. typhosus.*

Denys and Kaisin,[11] working along similar lines, found that the pleural exudates of rabbits, obtained by the injection of dead staphylococci and freed of cells by centrifugalization, were more highly bactericidal for staphylococci than the blood serum of the same animals, but found also that the inactivated exudate could not be reactivated by the addition of leukocytes. Denys offered as an explanation for these phenomena that the living leukocytes in the original exudate secreted alexin or complement which enhanced the bactericidal activity of the exudate, that the leukocytes, subsequently added to inactivated exudate, however, had lost vitality during the process of isolation and washing, and no longer possessed secretory power.

[9] Buchner. *Münch. med. Woch.*, No. 24, 1894.
[10] Hahn. *Archiv f. Hyg.*, Vol. 25, 1895.
[11] Denys and Kaisin, Denys and Havet. *La Cellule,* Vol. 9, 1893; Vol. 10, 1894.

Hankin,[12] Kanthack and Hardy [13] had gone even farther than this, and had attributed the production of alexin to the eosinophile leukocytes particularly.

Metchnikoff,[14] basing his opinion on his own studies, those of his pupils, and many other investigations similar to those mentioned above, came to the conclusion that, under ordinary conditions, the normal blood contains no free bactericidal substances. He assumes that these substances are entirely intracellular, being constituents of the various phagocytic elements, by means of which the cells digest the foreign elements they take up. He believes that there are essentially two varieties of such digestive enzymes or "cytases"—just as there are two varieties of phagocytes. The microphages, chiefly concerned in the digestion of bacteria, secrete the bactericidal alexin, or, as Metchnikoff calls it, "microcytase." The macrophages, the large mononuclear lymph and endothelial cells, primarily concerned in the phagocytosis of cellular elements (red cells, etc.) contain another variety of digestive enzyme, the "macrocytase," or cytolytic (hemolytic) alexin. The supposition that the hemolytic "cytase" is largely derived from the macrophages was based particularly upon the investigations of Metchnikoff's pupil, Tarassewitch,[15] who found that the extracts obtained from lymph nodes, and other organs rich in macrophages, possessed hemolytic properties. Both this work and the preceding studies regarding the extraction of alexin from polynuclear leukocytes will be more fully discussed below.

Maintaining that these cytases are purely intracellular under ordinary conditions, Metchnikoff believes that, in normal animals, the destruction of invading bacteria or of injected cellular substances (blood cells, etc.) is accomplished entirely by the phagocytic process, with subsequent intracellular digestion. In immunized animals, however, there is present in the circulating blood another substance, not identical with the cytases, but also derived from the leukocytes or from the blood-forming organs—the "fixateur" (Ehrlich's "amboceptor"—Bordet's "sensitizer"). This specific "fixateur" sensitizes the bacteria or other antigens to the action of the cytases. For his assumption regarding the origin of this sensitizer he finds support largely in the researches of Pfeiffer and Marx, and others mentioned in our section on the origin of antibodies, as well as in the similar investigations of Deutsch,[16] carried on under Metchnikoff's personal supervision.

Final digestion of the sensitized antigens (bacteria or blood cells), however, can take place only under the influence of the

[12] Hankin. *Centralbl. f. Bakt.,* Vol. 12, 1892.

[13] Kanthack and Hardy. *Proc. Roy. Soc.,* Vol. 52, 1892.

[14] Metchnikoff. *Ann. de l'Inst. Past.,* Vol. 7, 1893; Vol. 8, 1894; Vol. 9, 1895.

[15] Tarassewitch. *Ann. de l'Inst. Past.,* Vol. 16, 1902.

[16] Deutsch. *Ann. de l'Inst. Past.,* Vol. 13, 1899.

cytases intracellularly, unless by previous leukocytic injury these enzymes have been liberated into the blood stream.

While it is admitted, then, that bacteria may be killed and digested both intra- and extracellularly in the animal body, the cytases, which accomplish this, are regarded as the same in both cases, being derived from the phagocytic cells. In immunized animals "fixateur" may be produced under the stress of active immunization and furnished to the blood stream by the blood-forming organs. By this substance bacteria and cells may be sensitized. However, the enzyme by which digestion is actually accomplished, "cytase" or alexin, is not present free in the blood even in immune animals unless it has become free and extracellular by injury to the leukocytes.

How, then, on this basis does Metchnikoff account for the Pfeiffer phenomenon, in which the extracellular destruction of bacteria takes place rapidly in the peritoneal exudate? His explanation is the following: When bacteria or other substances are injected into the peritoneum there is a preliminary injury of leukocytes (phagolysis), and by this alexin or cytase is liberated. When cholera spirilla, for instance, are injected into the peritoneal cavity of an immunized guinea pig there follows a short period during which few if any leukocytes are present in the exudate, but many may be found gathered in motionless clumps in the folds of the peritoneum and mesentery, incapable of phagocytosis and apparently injured. If such leukocytic injury can be avoided Metchnikoff claims that the extracellular lysis of cholera spirilla will fail to take place. Thus if sterile broth or salt solution is injected into the peritoneum of a guinea pig a preliminary phagolysis will be followed by an accumulation of leukocytes. If cholera spirilla are now introduced no extracellular digestion is seen, but, instead of this, rapid phagocytosis takes place. This he says is due to the fact that the freshly accumulated, healthy phagocytes, collected in response to the preliminary broth injection, are not easily injured and do not undergo phagolysis; no cytase is liberated and, in consequence, no serum bacteriolysis can take place. In the same way he claims that the hemolysis of red blood cells (goose blood) in the peritoneum of specifically immunized guinea pigs may be prevented if, by a previous injection of broth, healthy leukocytes have been caused to accumulate. In such a case the goose blood corpuscles are rapidly ingested by the phagocytes and no hemolysis occurs.

It is self-evident that the validity of this interpretation of the occurrences is strictly dependent upon the demonstration that the circulating blood normally contains no alexin or complement. This is rigidly maintained by Metchnikoff, and is indeed one of the most important uncertainties of serology. He asserts that alexin appears in the blood serum only because changes in the leukocytes take place during coagulation. It is not, by any means, settled that Metchni-

koff is right in this—in fact, more recent investigations seem to show that he is wrong, and that we may assume definitely that alexin is present in considerable amounts in the circulating blood plasma of normal animals.

Metchnikoff's denial of this is based chiefly on the experiments of Gengou. Gengou [17] took the blood from various animals into paraffined tubes and centrifugalized it at low temperature before it could clot. This freed the plasma from the cells before clotting, though coagulation of course took place as soon as this plasma was removed to tubes and kept at room temperature. The serum expressed from this clotted plasma he compared for alexin contents (bactericidal properties) with that obtained from clotted whole blood.

He found that, in all cases examined (dogs, rabbits, and rats), the plasma contained practically no bactericidal substances, or at any rate an incomparably smaller amount than was present in the serum obtained from the clotted blood.

These experiments of Gengou would be conclusive in establishing Metchnikoff's theory if they were confirmed by other observers. This, however, has not been the case. Petterson [18] found no difference between the bactericidal properties of serum and oxalate plasma, and Lambotte [19] arrived at similar results when he compared serum with the coagulable plasma obtained by tying off a section of a vein and centrifugalizing the blood without opening the vessel. Hewlett,[20] Falloise,[21] Schneider,[22] and more recently Dick [23] and Addis,[24] whose work has been done with particular attention to technical accuracy, fail to confirm Gengou, finding no appreciable difference between plasma and serum.

In favor of Gengou's results are the investigations of Herman [25] and the more recent ones of Gurd.[26] Further supporting Gengou's conclusion is the observation recorded by a number of workers (Walker,[27] Longcope,[28] and others) that the complement or alexin contents of serum will increase somewhat as the serum is allowed to stand on the clot. This observation, too, has been rendered inconclusive by contrary reports from other investigators. Longcope,[29]

[17] Gengou. *Ann. de l'Inst. Past.*, Vol. 15, 1901.
[18] Petterson. *Arch. f. Hyg.*, Vol. 43, 1902.
[19] Lambotte. *Centralbl. f. Bakt.*, Vol. 34, 1903.
[20] Hewlett. *Zeitschr. f. Heilkunde*, 24, 1903.
[21] Falloise. *Bull. de l'Acad. Roy. de Méd.*, 1905.
[22] Schneider. *Arch. f. Hyg.*, 65, 1908.
[23] Dick. *Jour. Inf. Dis.*, Vol. 12, 1913.
[24] Addis. *Jour. Inf. Dis.*, Vol. 10, 1912.
[25] Herman. *Bull. de l'Acad. Roy. de Méd.*, 1904.
[26] Gurd. *Jour. Inf. Dis.*, Vol. 11, 1912.
[27] Walker. *Jour. Hyg.*, 3, 1903.
[28] Longcope. *Med. Bull. Univ. of Pa.*, 1902, Vol. 15, p. 331.
[29] Longcope. *Jour. Hyg.*, Vol. 3.

further, has found that alexin was more plentiful in the blood of individuals suffering from leukemia—in which of course a larger percentage of leukocytes is present in the circulation. This, too, has been contradicted by other workers, but even if upheld would not influence the possibility of there being alexin in the normal circulation. On the whole Gengou's contentions with their consequent bearing upon Metchnikoff's theory cannot be accepted as final. In fact, the greater part of available experimental evidence seems to point to the actual presence of alexin in the normal circulating blood. This seems also indicated by the unquestionable fact that active phagocytosis may take place in the circulation of an animal and, as we shall see below, free alexin is probably necessary (as opsonin) in this process. Further evidence in this direction also is furnished by the immediate anaphylactic shock which follows the injection of antigen into the blood stream of a sensitized animal, a process in which we have much reason to believe that alexin takes an active part. However, the problem is a difficult one, and, while we favor the opinion that free alexin is present in the intravascular blood, we must admit that a crucial experiment has not yet been formulated.

Leukocytic Bactericidal Substances.—Now, as regards the apparent extraction of alexin from leukocytes and lymphatic organs, we have already called attention to the fact that most of the researches associating these cells with the bactericidal substances were carried out before the dual mechanism of sensitizer and alexin in bacteriolysis had been fully worked out. In consequence conclusions were formulated from the mere facts of the presence of bactericidal or hemolytic properties in cell-extracts without the further determination of heat stability or the possibility of reactivation. Most of the earlier work also was done without sufficient attention to the separation of the cells and the serum of leukocytic exudates. The first one to do this carefully was Hahn,[30] who, like his predecessors, concluded that the bactericidal leukocytic substances, undoubtedly encountered by him, were identical with alexin. Doubt was first cast upon this by Schattenfroh,[31] who worked with leukocytes suspended and extracted in physiological salt solution. He found that bactericidal substances were, indeed, obtained in this way, but that, unlike alexin, these substances were relatively thermostable, withstanding exposure to a temperature of 56° C. and destroyed only by exposure to temperatures as high as 75° C. to 80° C. continued for thirty minutes.

Moxter,[32] a little later, working with cholera spirilla, also came to the conclusion that the leukocytic bactericidal substances were not

[30] Hahn. *Arch. f. Hyg.*, Vol. 25.
[31] Schattenfroh. *Arch. f. Hyg.*, Vols. 31 and 35, 1897.
[32] Moxter. *Deutsche med. Woch.*, 1899, p. 687.

identical with those found in the blood serum. Petterson,[33] too, made thorough investigations into the nature of the bactericidal substances extracted from the leukocytes, and, working chiefly with *B. proteus* and *B. anthracis,* found such substances in the leukocytes of dogs, rabbits, and guinea pigs active against the bacteria mentioned above, but failed to find them active, at least in guinea pig and cat leukocytes, against *B. typhosus* or the cholera spirillum. He expresses the opinion that bactericidal leukocytic substances are normally given up to the blood in minute quantity only or not at all, and that these substances hold no definite relationship to the bactericidal substances found in blood serum. In a later investigation he showed that the "endolysins," as he now calls the leukocytic bactericidal substances, may, like many enzymes and serum bacteriolysins, be precipitated out of solution with alcohol and ether; but he separates them absolutely from serum lysins and complement. The latter, while they may be, in part at least, secreted by the leukocytes, are, according to Petterson, induced easily to come out of the cells during life by slight injury or other stimulation, while the endolysins themselves are abstracted from the cells only after extensive extraction or maceration.

Schneider [34] has come to similar conclusions and speaks of the endocellular bactericidal substances as "Leukine." In a recent investigation of the same subject the writer [35] has in all essentials confirmed Schattenfroh's original conclusions regarding the heat stability of the extracted leukocytic bactericidal substances, and has shown that after inactivation by heat these substances are not reactivable by the addition of fresh leukocytic extracts, and that the yield obtained from the leukocytes of immunized animals is not greater than that obtained from normal leukocytes.

It appears, therefore, that, contrary to Metchnikoff's first supposition, the enzymes which bring about the digestion of phagocyted bacteria within the cell are not identical with those which bring about a similar extracellular digestion. In addition to the demonstration of a definitely different structure possessed by the bactericidal leukocytic extracts, as evidenced by their heat stability, we have the negative evidence that neither true alexin nor sensitizers have ever been successfully extracted from such cells.

It is still possible that this may eventually be done—but, although indirect evidence like that of Denys, Longcope's observations in leukemia, and the occasional increase of the alexic powers of serum after standing on the clot points to a probability of this, no direct evidence has so far been satisfactorily produced. In the hope that the leukocytes would give up alexin—possibly as a secretion as sug-

[33] Petterson. *Centralbl. f. Bakt.*, i, 39, 1905; 46, 1908.
[34] Schneider. *Archiv f. Hyg.*, Vol. 70, 1909.
[35] Zinsser. *Jour. Med. Res.*, Vol. 22, 1910.

gested by Denys—the writer, with Cary, some years ago kept washed leukocytes at 37.5° C. in Locke's solution, but was unable to find any evidence of alexin production within 48 hours.

The apparent extraction of hemolysin from macrophages by Tarassewith, moreover, has met with a similar refutation. Korschun and Morgenroth [36] have shown that these hemolytic extracts are extremely heat resistant, are alcohol and ether soluble, and do not act as antigens. They are quite different from the serum hemolysins, therefore, and probably closely related to the hemolytic lipoidal substances described by Noguchi and others.

Further strong arguments against the assumption of the presence of hemolytic alexin in the body of the macrophages have been advanced by Gruber [37] and by Neufeld.[38] Gruber showed that no extracellular hemolysis takes place when leukocytes are brought together with sensitized red blood cells, and Neufeld showed that even after the phagocytosis of such sensitized cells the hemolysis is very much slower, and of a different character from extracellular serum hemolysis. In the intracellular digestion there are no mere solution of the hemoglobin and formation of a shadow form (stroma), but there occur a gradual degeneration with the formation of a granular detritus of hemoglobin.

It is probable, then, that the digestion of bacteria and cells within the phagocytes is carried out by substances not identical with those taking part in serum lysis. It is not unlikely that the intracellular process is a quite complicated one, not depending on a single enzyme.

Leukoprotease.—In addition to the bactericidal substances extracted from leukocytes a number of true enzymes have indeed been obtained by various investigators. We have mentioned in another place that one of the earliest observations in this respect was that of Leber,[39] who noticed that sterile pus could liquefy gelatin. It may be commonly observed in paraffin or celloidin sections of staphylococcus abscesses that a ring of apparently digested or degenerating tissue is formed about an accumulation of leukocytes—in foci in which the bacteria may be too sparse to be held accountable for the changes. These leukoproteases have subsequently been carefully studied by Opie,[40] Jochmann and Müller,[41] and a number of others.

Opie found that two distinct proteolytic enzymes could be extracted from the cells of exudates obtained by turpentine injections. One—peculiar to the polynuclear leukocyte, and similar to one pre-

[36] Korschun and Morgenroth. *Berl. klin. Woch.*, 1902.

[37] Gruber. Quoted from Sachs, in "Kraus u. Levaditi Handbuch," Vol. 2, p. 991.

[38] Neufeld. *Arb. a. d. kais. Gesundheitsamt*, Vol. 28, 1908.

[39] Leber. "Die Entstehung der Entzündung," Leipzig, 1891.

[40] Opie. *Jour. Exp. Med.*, Vol. 7, 1905; Vol. 8, 1906; Vol. 9, 1907.

[41] Müller and Jochmann. *Münch. med. Woch.*, Nos. 29 and 31, 1906.

viously described by Müller [42]—acts in a weakly alkaline medium. The other, present particularly in exudates containing a predominating number of mononuclear cells, acts in a weakly acid reaction. Tschernorutski also found proteolytic ferments in both micro- and macrophages, but found no lipase in the polynuclear extracts. This seems particularly interesting in view of the great resistance to intracellular digestion noticed in acid-fast bacteria, a point of some importance in connection with the destruction in the body of such micro-organisms as the bacilli of tuberculosis and leprosy.[43] Jochmann [44] states that the action of the leukoprotease, which acts in an alkaline medium upon casein, results in the formation of tryptophan and ammonia, and believes it to be functionally very similar to trypsin. It is interesting to note that the most active protease is obtained from pus as it forms about acute infections or other stimuli which lead to the accumulation of polynuclear leukocytes, whereas it is apparently completely absent from tuberculous pus.

The question immediately arises, are these leukoproteases identical with the bactericidal substances extracted from leukocytes as described above? For it might well be that bacterial death resulted merely from the digestive action of the enzyme upon the bacterial protein. Jochmann,[45] who has approached this problem experimentally, has answered it in the negative. By repeated alcohol precipitation of glycerin extracts of leukocytes he obtained an enzyme preparation which possessed absolutely no bactericidal properties, though it was still actively proteolytic.

Not only did this relatively pure ferment possess no bactericidal action, but bacteria actively proliferated when suspended in it. Jochmann believes that living bacteria are not amenable to the enzyme possibly because of their possession of an "antiferment," at least this would follow in some cases from the experiments of Kantorowicz.[46]

The leukoproteases, therefore, appear to possess no direct significance in bacterial immunity. Their function seems rather to lie in the resorption of dead tissues, fibrin, blood clots, etc. Friedrich Müller [47] has pointed out their possible importance in the rapid destruction and liquefaction of the massive fibrinous exudates remaining after the crisis in lobar pneumonia.

Effects of Leukocytic Substances upon Infections.—From the discovery of antibacterial properties in the extracts of leukocytes it is but a logical step to the attempt to utilize these substances thera-

[42] Müller. *Kongr. f. inn. Med.*, Wiesbaden, 1902.
[43] Zinsser and Cary. *Jour. A. M. A.*, 1911.
[44] Jochmann. Leucozyten Fermente, etc., "Kolle u. Wassermann Handbuch," 2d Ed., Vol. 49, 2.
[45] Jochmann. *Zeitschr. f. Hyg.*, 61, 1908.
[46] Kantorowicz. *Münch. med. Woch.*, No. 28, 1909.
[47] Friedrich Müller. *Verhand. d. Kongr. f. inn. Med.*, 1902.

peutically. Petterson [48] was probably the first to study this phase of the problem systematically in connection with anthrax infection in dogs and rabbits. In preliminary studies he claimed to have determined that when leukocytes are left in contact with serum for four hours or longer there develops in the mixture a bactericidal power far superior to that which is possessed by these elements when separately kept in salt solution and mixed only just before the bactericidal tests. He attributes this to the fact that in dogs, at least, the leukocytes furnish bactericidal substances to the serum—an assumption which is entirely in accord with the earlier opinion of Denys and Kaisin,[49] which we have mentioned in another place. In direct continuance of these experiments he injected leukocytes into dogs at the same time at which he infected them with anthrax and observed a moderately protective influence, which, however, he admits was not very great. He followed this work in 1906 with similar observations on the protective influence of leukocytes in intraperitoneal infections of guinea pigs with typhoid bacilli. In these experiments [50] he made the curious observation that, although such protective influence was unquestionable, the guinea pig leukocytes contained no bactericidal substances active against typhoid bacilli. In consequence he concluded that the destruction of these bacteria in the guinea pig was due entirely to the serum-antibodies absorbed by the micro-organisms before phagocytosis, even when the actual destructive process took place intracellularly. The protective effect following on the injection of the leukocytes he attributed to an indirect influence of the leukocytic substances in stimulating the more rapid accumulation of alexin or complement in the peritoneum, with consequently more powerful phagocytosis. Following this, in 1908, Opie [51] carried out experiments in which he observed that leukocytes injected intrapleurally into dogs, together with tubercle bacilli, exerted a distinct protection.

In the same year extensive observations on the protective properties of leukocyte extracts were published by Hiss.

Hiss [52] worked at first with extracts of dog, rabbit, and guinea pig leukocytes; later he confined himself entirely to rabbit leukocytes. He extracted the leukocytes at first by repeated freezing and thawing in physiological salt solution, but the technique of his subsequent work was uniformly as follows: Intrapleural injections of aleuronat emulsions were made in rabbits and, after about 24 hours, the resulting exudates were taken away with sterile pipettes and centrifugalized before clotting could take place; the serum was de-

[48] Petterson. *Centralbl. f. Bakt.,* Vol. 36, 1904.
[49] Denys and Kaisin. "La Cellule," Vol. 9, 1893.
[50] Petterson. *Centralbl. f. Bakt.,* Vols. 40 and 42, 1906.
[51] Opie. *Jour. Exp. Med.,* 1908.
[52] Hiss. *Jour. Med. Res.,* new series, Vol. 14, 1908.

canted and the leukocytes then emulsified in distilled water, in quantity about equal to the amount of serum poured off. In this the leukocytes were allowed to stand for a few hours at incubator temperature, and then in the ice-box until used. For his experimental work in both animals and man, in most instances, not only the clear supernatant fluid was injected, but the cell residue as well.

With leukocytic extracts so prepared Hiss treated staphylococcus, typhoid bacillus, pneumococcus, streptococcus, and meningococcus infections in rabbits and obtained results which justified him in concluding that the leukocyte extract exerted strong protective action in all of these cases. Many of his animals survived infections fatal to controls even when the treatment was delayed as long as 24 hours after infection. Subsequently Hiss and Zinsser [53] treated series of patients, ill with pneumonia, meningitis, and staphylococcus infections, with leukocyte extracts prepared by the method of Hiss, and felt that they were justified in concluding that in many cases, at least, the course of the disease was favorably influenced by the leukocyte extract. Favorable results have since then been obtained also by Lambert in erysipelas, and by Hiss and Dwyer in a variety of conditions.

While there seems to be little question about the actually favorable influence of the leukocyte extract, both in experiments with animals and in the treatment of human cases, there has been considerable difficulty in determining the reasons for this influence. In subsequent studies Hiss and Zinsser (*loc. cit.*) were able to show that the extracts did not favor phagocytosis and that the moderate bactericidal properties possessed by the leukocytic substances could not account for their effectiveness. There *did* seem to be a more rapid accumulation of phagocytes in the peritoneal cavities of guinea pigs infected with cholera spirilla when leukocyte extract was injected with the bacteria, and it is not impossible, in fact, it seems probable to the writer, from subsequent experience, that the protective properties of the leukocyte extracts are attributable, in part at least, to their positively chemotactic effect.

We are inclined to believe at present that the beneficial effects of leukocyte extracts are based on the same principles as those which determine the reactions following on the injection of bacterial and any other protein.

The Problem of Leukocytosis.—In this connection a very interesting problem has arisen—namely, that spoken of as the phenomenon of *Specific Hyperleukocytosis.* Bordet, as early as 1896, made the following statement, "Active immunity has also other characteristics, in that there is an increase of the number of leukocytes, that is, an 'exaltation' of the chemotactic sensibilities of the leukocytes." He suggests herein that an immunized animal may respond with a more

[53] Hiss and Zinsser. *Jour. Med. Res.*, new series, Vol. 14, 1908.

powerful leukocytic reaction to the injection of the infectious agent than would a normal animal similarly treated. This idea has recently found experimental elaboration in the work of Gay and Claypole, who found that the reinjection of immune animals with the homologous bacteria produced a specific hyperleukocytosis, that is, typhoid immune animals receiving typhoid bacilli would respond with counts ranging up to 150,000 leukocytes per cu. mm., whereas the normal animals rarely showed more than 40,000 to 50,000.

This observation would tend to indicate a great advantage of the specific over the non-specific methods of treatment.

Unfortunately the results of Gay and Claypole [54] have not found confirmation. McWilliams [55] in similar experiments found no differences in the degree of leukocytosis between normal and immune animals in response to the injection of bacteria and reported that the same degree of response followed in typhoid immune animals when injected with *Bacillus coli* as when typhoid bacilli were administered. In part, this is also stated to be the experience of Jobling and Petersen.

The question is such an important one that the writer, with Dr. Tsen,[56] reinvestigated it in connection with work on the therapeutic effect of leukocytic extract. Our conclusions showed little agreement with those of Gay and Claypole.

We found that when homologous Gram-negative bacilli are injected into immunized animals there seems to be a definitely higher leukocytosis in the immunized animals than in normal controls similarly treated. The contrasts in our experiments, however, were nothing like as striking as those reported by Gay and Claypole. Indeed the contrast in general is so slight and so irregular that in the case of the Gram-negative bacilli we were at first inclined to agree with McWilliams. There was, however, a sufficiently definite difference in an average of many counts to convince us that this was more than coincidence.

In the case of the Gram-positive cocci there was a more marked difference, in that the immunized animals reacted more promptly and very much more energetically than did the normal animals to similar injections.

It seems reasonably clear, then, that an animal reacts more energetically as far as its mobilization of leukocytes is concerned when reinjected than does a normal animal treated with the same variety and quantity of bacteria.

The reaction is dependent upon a number of factors, chief among which are: (1) the condition of the animal (loss of weight, etc.); (2) the amount of bacteria injected, and (3) the interval between

[54] Gay and Claypole. *Arch. of Int. Med.,* V, XIV, 1914, p. 662.
[55] McWilliams. *Jour. Immunol.,* Vol. I, No. 2, 1916, p. 159.
[56] Zinsser and Tsen. *Jour. Immunol.,* Vol. II, No. 3, 1917, p. 247.

injections. These factors all very naturally signify the necessity of avoiding too profound an intoxication of the animal.

When immune animals are treated with heterologous bacteria—that is, when prodigiosus bacilli or colon bacilli are injected into typhoid immune animals and vice versa—there seems to be no specific difference in response. That is, the injection of colon bacilli into typhoid animals has shown no marked difference in leukocytic response from that observed when typhoid bacilli were injected into a typhoid animal. In this our figures correspond with those of McWilliams. They are also in keeping with the clinical experience of Kraus, Ichikawa, and others which have been mentioned above.

The injection of leukocytic extract does not arouse as vigorous a leukocytic response as does the injection of bacillary protein.

In reading these facts superficially they at first seem to be contradictory in significance, inasmuch as specificity seems to exist in the fact that typhoid immune rabbits or streptococcus immune rabbits respond somewhat more vigorously than do normal controls injected with the same substance.

However, we think that these relations are explained by the fact that animals that have reacted to such organisms as the typhoid bacillus, etc., develop a certain amount of non-specific tolerance against the proteose-like substances which are probably responsible for a not unimportant part of the symptoms caused by the bacteria. Such tolerance has been shown in the experiments made by the writer with Dwyer.

To summarize, therefore, we do not think that at present a specific leukocytosis in the sense of Gay and Claypole has been demonstrated, but believe that an animal, immune to one micro-organism, will have a slight non-specific increase of resistance to other organisms.

This does not mean that the immunity is non-specific. The destruction of living bacteria is still a purely specific process, and this, of course, would determine the occurrence of outcome of an infection.

CHAPTER XV

FACTORS DETERMINING PHAGOCYTOSIS

OPSONINS, TROPINS AND THE OPSONIC INDEX

From the very beginnings of his researches upon phagocytosis Metchnikoff recognized that the process was profoundly influenced by the properties of the fluid constituents of the blood plasma in which the phenomenon occurred. Both he and his pupil Bordet,[1] at this time working in Metchnikoff's laboratory, noticed that the phagocytic activity of leukocytes was greater in immune than in normal sera and associated this with the specific properties of the immune substances or antibodies in these sera; Metchnikoff himself interpreted the phagocytosis-enhancing power of the serum as a stimulation of the leukocytes and referred to the serum constituents by which this effect was produced as "stimulins." A closer analysis of the factors involved in this interrelationship, however, was not attempted at this time by him or his pupils, although indirect reference was made to it in a number of articles emanating from this school in the course of investigations on kindred problems of phagocytosis. Thus Gabritschewsky,[2] in 1894, published a paper on "Leukocytose dans la Diphthérie," in which he concluded that the poison of diphtheria bacilli, among other harmful effects, diminished the phagocytic power of the leukocytes, and that one of the beneficial influences of the curative serum was to render these and other cells "less sensitive to the bacterial poisons." This may be interpreted as indicating an assumption that the action of an immune serum in increasing phagocytic activity rested rather upon its influence upon the bacterial products than upon any stimulation of the phagocytes themselves. However, in diphtheria the action of the leukocytes was, even at this time, recognized as a merely secondary one, and Gabritschewsky's results did not materially influence the "stimulin" conception.

The first extensive investigation which occupied itself directly with these problems was that of the Belgian bacteriologists Denys and Leclef.[3] The publication of these workers deals primarily with the nature of streptococcus immunity in rabbits. It established, first

[1] Bordet. *Ann. de l'Inst. Past.*, 1895.
[2] Gabritschewsky. *Ann. de l'Inst. Past.*, 1894.
[3] Denys and Leclef. *La Cellule,* 11, 1895.

of all, the paramount importance of phagocytosis in the resistance of animals against these bacteria, and made clear that the destruction of bacteria was carried out equally as well by the leukocytes of normal as by those of immune animals, but was powerfully enhanced when either normal or "immune" leukocytes were combined with immune serum. Their work, therefore, indicated again that the increased phagocytosis of virulent bacteria, taking place in immune animals, depended clearly upon alterations in the functions of the serum rather than in those of the cells, and they suggested that the influence of this serum was not necessarily one of leukocyte stimulation, but might rather consist in action upon the bacteria, rendering them less resistant to phagocytosis. They say in substance: "A notre avis, on pourrait tout aussi bien admettre que la substance vaccinante ou antitoxique agit, non pas sur le leukocyte, mais sur un poison renfermé dans le corps du microbe ou dissous dans le milieu, et qui préserve le micro-organisme contre les atteintes du leukocyte." [4]

In this statement we have, in brief, the distinct formulation of our present view of the "opsonins." [5]

Observations with pneumococci and streptococci carried out after this by Marchand [6] and by Mennes,[7] whose investigations we cannot discuss in detail, beside confirming most of the observations of Denys and Leclef, brought out especially the relation of the virulence of micro-organisms to phagocytosis, showing that very virulent strains were taken up to a slight degree only in the presence of normal serum, but were subject to active phagocytosis when immune serum was employed. This, too, seemed to point primarily to the fact that the serum influenced rather the bacteria than the phagocytes, although no convincing proof is brought for this in their publications. Though much that had bearing indirectly on this problem was written during the following years, no definite progress was made beyond the results of Denys and his pupils until 1902, when Leischman [8] introduced a technique by means of which it be-

[4] In our opinion one can just as well believe that the vaccinating or antitoxic substance acts not upon the leukocyte but upon a poison inclosed within the body of the bacteria or dissolved in the medium, which preserves the micro-organism against the attacks of the leukocyte.

[5] Denys formulated this view with still greater clearness and positiveness at the Congress of Hygiene held at Brussels in 1903. We take our citation from the discussion on opsonins by Gruber (3d meeting Freie Vereinigung f. Mikrobiol., Vienna, 1909, *Centralbl. f. Bakt.*, I Ref., Vol. 44, Suppl. p. 3). Following is Denys' statement: 1. The phagocytosis in immune sera is dependent upon substances which are precipitated with the euglobulins. 2. These substances cause phagocytosis by inciting a physical alteration of the micro-organisms. 3. These substances are specific.

[6] Marchand. *Arch. de Méd. Exp.*, 1898.

[7] Mennes. *Zeitschr. f. Hyg.*, Vol. 25.

[8] Leischman. *Brit. Med. Jour.*, Vol. 2, 1901, and Vol. 1, 1902.

came possible to observe the process of phagocytosis with fresh serum and leukocytes *in vitro*.

By utilizing this technique and improving upon it Wright and Douglas in the following year (1903) evolved a method by means of which phagocytic activity could be quantitatively measured with reasonable accuracy. They worked at first with staphylococcus phagocytosis by human leukocytes in the presence of human citrate plasma, a research undertaken primarily because Wright,[9] in collaboration with Windsor, had previously determined that human blood serum possessed practically no bactericidal power for this organism, and that phagocytosis was probably the chief mechanism of protection which the human body possessed against these bacteria. The researches of Wright and Douglas [10] were carried out chiefly by mixing equal volumes of bacteria, serum, and leukocytes (in citrate suspension),[11] allowing these elements to remain together at 37.5° C. for varying periods, then staining on slides and determining the degree of phagocytosis by counting the numbers of bacteria taken up by each polynuclear leukocyte. Though many technical difficulties had to be overcome, and although the method at its best still permits of much personal error, careful work and untiring repetition made possible a considerable degree of accuracy, and definite facts regarding the mechanism of phagocytosis, heretofore merely suspected, could be recorded. The most important result of these investigations was the unquestionable establishment of the function of serum in the process of phagocytosis, namely, that it in no way "stimulated" the leukocytes in the sense of Metchnikoff, but rather acted entirely upon the bacteria, preparing them for ingestion. For this reason Wright coined the word "opsonins" (*ὀψονέω* = I prepare food) for the serum constituents which brought about this effect, believing them to be new antibodies, entirely distinct from the other serum antibodies heretofore recognized.

Wright and his followers now concluded that the rôle of the leukocyte in taking up bacteria was entirely dependent upon the opsonin contents of the serum. In a menstruum containing no serum, or in a serum in which the opsonins had been destroyed by heat, they found practically no phagocytic action on the part of washed serum-free leukocytes, and they, therefore, doubted the occurrence of spontaneous phagocytosis on the part of leukocytes themselves.

In this point it is not unlikely that Wright is mistaken, since

[9] Wright and Windsor. *Jour. Hyg.*, Vol. 2, 1902.

[10] Wright and Douglas. *Proc. Roy. Soc.*, 72, 1903, 73 and 74, 1904. See also Wright, "Studien über Immunisierung," Fischer, Jena, 1909.

[11] At first bacteria were merely mixed in equal volumes with citrated whole blood.

other observers, notably Löhlein,[12] have observed the phagocytosis of various bacteria by washed leukocytes in indifferent, opsonin-free media. Although we may take it as assured that such spontaneous phagocytosis may take place (Metchnikoff and a number of others having obtained results similar to those of Löhlein), this is probably never very intense.

In fact, Wright, in some of his later work, does not insist rigidly upon the non-occurrence of spontaneous phagocytosis, but attempts to associate such phenomena with the salt contents of the medium in which it occurs. Together with Reid,[13] he determined that spontaneous phagocytosis of tubercle bacilli unquestionably takes place, is most intense at a concentration of about 0.6 per cent. NaCl, and diminishes as the concentration is increased. This, as we shall see, has bearing on the possible physical explanations advanced to account for opsonic action, and has its parallels in experiments on the influence of electrolytes on agglutination and precipitation.

The fact remains that Wright demonstrated by his work that Metchnikoff's original view, which interpreted the difference between susceptibility and immunity as a difference between the inherent phagocytic powers of the leukocytes, is incorrect, and that the essential regulating influence affecting phagocytosis rests upon the action of the serum upon the bacteria.

The following experiment from the work of Hektoen and Ruediger [14] illustrates this point with exceptional clearness. It shows that human leukocytes in the presence of normal defibrinated blood will take up bacteria energetically. When the leukocytes, however, are washed free of blood and added to untreated bacteria phagocytosis is practically nil. If, however, such washed leukocytes are mixed with bacteria that have been previously in contact with serum active phagocytosis will take place. In other words, the bacteria have been altered by the serum in such a way that they are now amenable to phagocytosis by washed leukocytes. The serum then acts upon the bacteria and not upon the leukocytes.

TABLE II

Phagocytosis by Human Leukocytes of Sensitized Bacteria

	Average Phagocytosis
Human leukocytes (defibrinated blood) + Staphylococcus aureus . . .	22.
Human leukocytes (washed in NaCl solution) + Staphylococcus aureus . . .	1.2
Human leukocytes (washed in NaCl solution) + Staphylococcus aureus (treated with human serum) . . .	10.
Human leukocytes (defibrinated blood) + Streptococcus 300 . . .	22.

[12] Löhlein. *Centralbl. f. Bakt.*, 38, 1906, Beiheft, p. 32; also *Münch. med. Woch.*, 1907, p. 1473.

[13] Wright and Reid. *Proc. of Roy. Soc. B.*, Vol. 77, 1906.

[14] Hektoen and Ruediger. *Jour. Inf. Dis.*, Vol. 2, 1905, p. 132.

	Average Phagocytosis
Human leukocytes (washed in NaCl solution) + Streptococcus 300..	1.
Human leukocytes (washed in NaCl solution) + Streptococcus (treated with human serum)...........................	14.
Human leukocytes (washed in NaCl solution) + Streptococcus (treated with guinea pig serum)...........................	12.
Human leukocytes (washed in NaCl solution) + Streptococcus (treated with rabbit serum)...........................	14.

Wright and Douglas'[15] work was done at first with normal serum or normal citrate plasma, and in this case they found that the opsonins were essentially unstable, being easily weakened by exposure to light, or heat, and even when preserved in sealed tubes in the dark they diminished noticeably on standing for 5 or 6 days. Other writers who have worked with the opsonic substances in normal serum have confirmed this instability of the normal opsonin, although even Wright himself admits that heating to 60° C. does not entirely destroy the opsonic power, though it reduces it to a minimum. A protocol from Wright and Douglas' first paper will best illustrate the degree of reduction of opsonic power resulting from the exposure of normal serum to 60° to 65° C. for 10 to 15 minutes.

A. Unheated serum Wright—Staphylococcus suspension 1 vol.—Blood cells Wright 3 vols.

(1) Phagocytic average 20 cells..............	17.4
(2) Phagocytic average 20 cells..............	19.8

B. Heated serum as above.

(1) Phagocytic average 52 cells..............	0.6
(2) Phagocytic average 46 cells..............	3.4

The experiments just cited refer only to the opsonic powers of normal serum. When an animal is immunized with any particular micro-organism or other cellular antigen, such as red blood cells, etc., a marked specific increase of opsonins occurs, but unlike the opsonins of normal serum these newly formed elements in the immune serum seem to possess a much greater resistance to heat.

Neufeld and Rimpau,[16] who have studied these constituents of immune serum with especial thoroughness, have shown that heating to 62° to 63° C. for as long as three-quarters of an hour does not destroy them, and that such sera may be preserved for as long as several years without their complete disappearance.[17]

We may accept as definitely determined, therefore, that there is a qualitative difference between the serum components which initiate

[15] Wright and Douglas. Cited in Wright, "Studien über Immun., etc.," p. 9.

[16] Neufeld and Rimpau. *Deutsche med. Woch.*, No. 40, 1904; *Zeitschr. f. Hyg.*, Vol. 51, 1905.

[17] Leishman. *Trans. London Path. Soc.*, Vol. 56, 1905.

phagocytosis in normal serum (normal opsonins) and those which carry out the same function to a much more powerful degree in immune serum. This is the more surprising since, in the case of all other antibodies (lysins, agglutinins, etc.), it has been shown that in structure and mode of action the antibodies of immune serum are in every way qualitatively similar to the corresponding ones of normal serum,[18] representing merely a specific quantitative increase of substances originally present in small amount.

This difference between the normal and immune opsonic substances has added much difficulty to the investigation of the nature of these bodies, and we may approach the problem with greater clearness by considering them separately, at first, attempting to define the relations between them after we have set down the facts ascertained in connection with each.

Relation of Opsonins to Alexin and Other Antibodies.—In their earliest investigations upon the normal opsonins Wright and Douglas [19] regarded them as new antibodies, separate and distinct from those already known. There is no convincing proof of this, and a number of other interpretations of the observed phenomena are possible. Indeed, the burden of proof is rather upon those who would establish the existence of a new antibody, for before this can be done it must be shown that the new function is not merely another property of the serum constituents already known. For, as Gruber has justly said, "One of the most important attributes of the natural scientist is economy of hypotheses." And in the case of the normal opsonins there are many good reasons for regarding them as possibly identical with known serum constituents. The two possibilities suggested have been (1) Are the opsonic substances identical with the alexin or complement? or (2) Do they represent the combined action of the normal sensitizer of the serum activated by the alexin?

The similarity of normal opsonin with alexin or complement has been brought out especially by Muir and Martin,[20] by Baecher,[21] and by Levaditi and Inmann.[22] The fact that both are thermolabile has been mentioned above.

In addition to this, as Muir and Martin [23] have shown, all antigen-antibody complexes which absorb alexin out of serum at the same time remove the normal opsonin. Thus sensitized red corpuscles,

[18] Dean. *Proc. Roy. Soc.*, 76, 1905. Neufeld and Hüne. *Arb. a. d. kais. Gesundh. Amt.*, Vol. 25, 1907.

[19] Wright and Douglas. *Loc. cit.*

[20] Muir and Martin. *Brit. Med. Jour.*, Vol. 2, 1906; *Proc. Roy. Soc. B.*, Vol. 79, 1907.

[21] Baecher. *Zeitschr. f. Hyg.*, Vol. 56, 1907.

[22] Levaditi and Inmann. *C. R. de la Soc. Biol.*, 1907, pp. 683, 725, 817, 869.

[23] Muir and Martin. *Loc. cit.*

sensitized bacteria, and specific precipitates added to normal serum take out its opsonic substances. From this fact they also concluded that the normal opsonins like alexin were non-specific. For just as the alexin of a serum may serve to activate a considerable variety of sensitized antigens, so the opsonic action of a normal serum may functionate upon a large variety of bacteria. Muir and Martin were probably wrong in this and, as we shall see below, normal opsonins, like normal sensitizers, may be regarded as specific.

Similar to the observations of Muir and Martin are those of Neufeld and Hüne,[24] which showed that yeast cells will absorb both alexin and opsonin out of serum.

A further similarity between the two serum constituents is the fact that both are absent from the normal fluid of the anterior chamber of the eye, but they together appear in it after injury (puncture for the first removal of fluid). A like parallelism between the absence and presence of both has been shown for edema fluids. Furthermore, phosphorus poisoning which reduces alexin likewise reduces opsonin.

Although this parallelism is very striking, it does not on this account mean that necessarily the two are identical. It may signify merely that the alexin is a necessary participant in normal opsonic action, essential in that it activates a thermostable opsonic constituent just as it activates hemolytic or bactericidal sensitizer.

This opinion has been expressed by Levaditi, Neufeld,[25] Dean,[26] and others, and indeed it is a conception which seems most logical. For the procedures which remove both alexin and opsonin, as stated above, do not, as a matter of fact, remove *all* the opsonic action. (Although Neufeld maintains this.[27]) Studies of Hektoen and others have definitely proved that, though reduced to almost nil, nevertheless heated serum shows definite though slight opsonic action as compared with indifferent menstrua such as salt solution. A similar slight remnant of opsonic action after absorption of normal serum with sensitized cells, bacteria, and precipitates is evident in the protocols of Muir and Martin. The significance of this point becomes immediately clear when we consider the properties of the bacteriotropins or immune opsonins, which are heat stable and capable of initiating opsonic action in the entire absence of alexin or complement. It is possible, therefore, that there may be present in normal serum a slight amount of specific thermostable opsonin, which, though capable of acting feebly by itself, is nevertheless powerfully

[24] Neufeld and Hüne. *Arb. a. d. kais. Gesundh. Amt.*, Vol. 25, 1907.
[25] Neufeld. "Kolle u. Wassermann's Handbuch," Ergänzungsband 2, p. 313.
[26] Dean. *Brit. Med. Jour.*, 2, 1907, p. 1409.
[27] In fact he states that heated normal serum may be used as a control in opsonic experiments instead of salt solution.

activated by alexin—just as bactericidal or hemolytic antibody is similarly activated.

One of the most thorough studies upon this question is that of Cowie and Chapin.[28] Dean [29] had previously shown that, although heated immune serum was capable of exerting opsonic action by itself, this action could nevertheless be enhanced by the addition of a little diluted fresh normal serum. The particular significance of Dean's work will be discussed later. Cowie and Chapin, however, carried on similar experiments with normal serum in which they attempted to reactivate heated normal serum by the addition of small amounts of diluted fresh serum, by itself but slightly opsonic. One of their experiments may serve to illustrate this point, as follows:

Experiment 10. June 13, 1907

	Phagocytic count *
1. Unheated serum	15.44
2. Salt solution	0.18
3. Heated serum, 57° C.	1.08
4. Diluted serum (1:15)	1.56
5. Heated serum 57° C. + diluted serum (1:15)	12.40
6. Unheated serum + unheated serum	16.08

* Phagocytic count = average number of bacteria in each leukocyte.

This experiment and others like it seem to demonstrate clearly that the opsonic action of normal serum, though dependent largely upon alexin, is nevertheless also dependent upon a heat-stable body, comparable to the sensitizer or amboceptor, in that it is reactivable to almost the full power of the original condition (before heating) by slight amounts of alexin—in themselves almost inactive.[30]

These findings were later confirmed by Eggers,[31] and it is plain from this work that the apparent opsonic inactivation of normal serum by heat depends upon the destruction of the heat-sensitive constituent only—the heat-stable substance—surely involved in the process, remaining intact, and reactivable.

Closely associated with this phase of the problem is that of the specificity of the normal opsonins. For if, as at first supposed, the normal opsonins are, like complement or alexin, non-specific, the above amboceptor-complement structure of this mechanism would be rendered unlikely. Earlier work upon this question was con-

[28] Cowie and Chapin. *Jour. Med. Res.*, Vol. 17, 1907, pp. 57, 95 and 213.

[29] Dean. *Loc. cit.*

[30] In earlier experiments Hektoen and Ruediger [33] did not succeed in reactivating heated sera and concluded that normal opsonins had the hypothetical structure of toxins in that they possessed a haptophore and an opsonophore group. From this point of view Hektoen has subsequently receded largely because of work done under his own direction.

[31] Eggers. *Jour. Inf. Dis.*, Vol. 5, 1908.

tradictory. Bulloch and Western,[32] working with staphylococci and tubercle bacilli, found that each of these organisms absorbed out separately specific opsonins from normal serum, leaving those for other bacteria but slightly reduced. Slight reduction of the opsonic action for other micro-organisms might easily be explained by a partial removal of complement which is bound to take place in such experiments. Simon, Lamar and Bispham,[33] and some others failed to find any such specificity. Russell,[34] Axamit and Tsuda,[35] and a number of others obtained similar negative results—in that a number of different bacteria seemed to absorb opsonins out of normal serum indiscriminately and without specificity. On the other hand, more recent careful work by Rosenow,[36] by Macdonald,[37] and by Hektoen [38] has upheld the original contention of Bulloch and Western. The work of Rosenow, in which pneumococci were shown to absorb out their specific opsonins from normal human serum, taking out in part only those for streptococci, staphylococci, and tubercle bacilli, is especially convincing, and the experiment of Hektoen with normal hemopsonins (opsonins which cause the phagocytosis of red blood cells) bear him out.

It seems fair to conclude, therefore, that normal opsonins depend upon the coöperation of a heat-stable and a heat-sensitive body. The heat-stable body, analogous to normal sensitizer or amboceptor, is specific and reactivable by the heat-sensitive body which appears to be identical with alexin. This statement merely asserts the facts of the dual mechanism of the process without assuming necessarily the identity of the heat-stable body with sensitizer or that of the heat-sensitive one with alexin, though this seems extremely probable.

This question we will discuss again more particularly in connection with the bacteriotropins or immune opsonins.

Further proof for such a complex constitution of the normal opsonins has been adduced by means of absorption experiments at 0° C.—by Cowie and Chapin. In our discussion of the lytic antibodies we have seen that sensitizer or amboceptor may be absorbed from serum by its specific antigen at 0° C.—but that the attachment of alexin takes place only when the temperature is raised above this. Practically no alexin is bound at the low temperature. Cowie and Chapin, applying this method of investigation, showed:

1. That normal human serum may have its opsonic power for staphylococci removed by absorption with staphylococci at 0° C.

[32] Bulloch and Western. *Proc. Roy. Soc. B.*, 77, 1906.
[33] Simon, Lamar, and Bispham. *Jour. Exp. Med.*, Vol. 8, 1906.
[34] Russell. *Johns Hopkins Bull.*, Vol. 18, 1907.
[35] Axamit and Tsuda. *Wien. klin. Woch.*, Vol. 20, No. 35, 1907.
[36] Rosenow. *Jour. Inf. Dis.*, Vol. 4, 1907.
[37] Macdonald. Quoted from Hektoen, *loc. cit.; Aberdeen Univ. Studies*, Vol. 21, 1906, p. 323.
[38] Hektoen. *Jour. Inf. Dis.*, Vol. 5, 1908.

2. Serum so treated retains the power of reactivating the opsonin of heated normal serum.

3. Staphylococci so treated are more easily subject to phagocytosis in the presence of dilute normal serum, or normal serum which has been inactivated by contact with staphylococci in the cold, than are the same bacteria untreated.

Kurt Meyer [39] has carried out similar experiments with paratyphoid bacilli and normal serum, and, though his work is less extensive, he reaches the same conclusion as Cowie and Chapin.

We may accept, therefore, as fairly well established that the opsonic power of normal serum depends upon a complex mechanism consisting of (a) a thermostable substance comparable to amboceptor or sensitizer, probably specific, but present in very small amount, and (b) a thermolabile substance probably identical with alexin or complement which powerfully, but non-specifically, enhances the slight opsonic power of the thermostable substance.

In considering this conception, together with the subsequent discussion of bacteriotropins or immune opsonins, it will be well to remember that in normal inactivated sera the thermostable opsonic constituent differs in its action from the bodies we speak of as amboceptors or sensitizers in that it may functionate for phagocytosis by itself—entirely without alexin—while neither bactericidal nor hemolytic effects can be brought about by sensitizer alone. Does this definitely exclude the identity of this thermostable opsonic substance and sensitizer? It is indeed an argument against identification, but in opsonic action, we must remember, there is merely a sensitization to the action of the phagocyte. This phagocyte may in itself be capable of furnishing a small amount of substance comparable in action to alexin—in fact, we have seen that the origin of alexin from leukocytes is still suspected by a number of workers. At any rate the phagocyte is a living cell which may well be capable of supplying in itself to some degree the necessary activation, and therefore the difference cited above is not necessarily a proof that the normal thermostable opsonic constituent is different from normal sensitizer or amboceptor.

The difference between the opsonic action of normal serum and that of immune serum, then, is the fact that heating to from 56° to 60° C. almost completely destroys the former, whereas it has but slight if any diminishing effect upon the latter. The immune opsonins, or, as Neufeld and Rimpau have called them, bacteriotropins, therefore are thermostable. This was determined as early as 1902 by Sawtschenko,[40] and was subsequently studied with great accuracy

[39] Kurt Meyer. *Berl. klin. Woch.*, 1908, p. 951.
[40] Sawtschenko. *Ann. de l'Inst. Past.*, Vol. 16, 1902, quoted from Levaditi.

by Neufeld and Rimpau,[41] Neufeld and Töpfer,[42] Dean,[43] Hektoen,[44] and others. It was shown that when an animal is immunized with any given bacterium or other cellular antigen (blood corpuscles, etc.) opsonic substances specific for the particular antigen appear in considerable quantities, and these are but slightly, if at all, diminished when the serum is heated; Neufeld and Hüne[45] found that heating for as long as three-quarters of an hour to 63° C. did not noticeably reduce the activity of the bacteriotropins of immune serum, and that, again, unlike the normal opsonins, prolonged preservation, under sterile conditions, changes them but slowly.

These facts alone indicate a close similarity between the bacteriotropins and the other well-known thermostable constituents of immune sera, and the question here again immediately arises whether we are to regard them as identical with any of the other specific antibodies or as distinct substances independent of these.

It was suggested early in these investigations by Muir and Martin that bacteriotropins might be identified with agglutinins, inasmuch as they possessed resistance to heat, were active without apparent dependence upon alexin, and could not, at least in the earlier studies, be reactivated by the addition of fresh normal serum when once inactivated. The supposition was that for this reason the bacteriotropin might have a structure like the hypothetical "haptines of the second order" which Ehrlich attributes to the agglutinins. This supposition has found no experimental support in that agglutination and bacteriotropic effects did not run parallel. We ourselves are not ready to admit that such lack of parallelism is proof against their identity. However, since it is very probable that both agglutination and precipitation are merely phenomena of colloidal flocculation effects which follow certain quantitatively adjusted combinations of antigen and specific antibody, and that it is not at all necessary to assume separate agglutinating or precipitating serum constituents, this problem becomes merely another version of the question of the identity of bacteriotropins and sensitizer or amboceptor.

Apart from thermostability, further similarity lies in the fact that bacteriotropins are strictly specific and may be specifically absorbed out of immune sera by their respective bacteria.

Like amboceptor or sensitizer they are specifically increased to a powerful degree by the treatment of animals with any given microorganism and may be incited not only by the injection of bacteria but by that of blood cells as well. In spite of these points of likeness,

[41] Neufeld and Rimpau. *Deutsche med. Woch.*, No. 40, 1904; *Zeitschr. f. Hyg.*, 51, 1905.
[42] Neufeld and Töpfer. *Centralbl. f. Bakt.*, 1, 38, 1905.
[43] Dean. *Proc. Roy. Soc. B.*, 76, 1905.
[44] Hektoen. *Jour. Inf. Dis.*, 3, 1906, and *loc. cit.*
[45] Neufeld and Hüne. *Arb. a. d. kais. Gesundh. Amt.*, Vol. 25, 1907.

however, Neufeld [46] and his associates maintain rigidly that the two substances are not the same and that the bacteriotropins are distinct and independent antibodies.

Among the reasons advanced in support of this opinion are the facts that certain immune sera, both antibacterial and hemolytic, may contain bacteriotropins without containing lysins and vice versa. That this is undoubtedly true has been shown not only by Neufeld and his associates but by Hektoen [47] and others, and it is likewise a fact that in sera in which both functions are demonstrable they frequently do not run quantitatively parallel. These are unquestionably strong arguments, but their force is somewhat weakened, as Levaditi has pointed out, by the fact that there are many varieties of bacterial immune sera which undoubtedly sensitize the specific bacteria (as can be shown by alexin fixation), but which do not lead to bacteriolysis. Wassermann [48] also attaches little value to the lack of parallelism between the lytic and opsonic functions, expressing the belief that the solubility of the particular antigen may determine whether sensitization leads to phagocytosis or to lysis. With bacteria like the cholera spirillum rapid lysis takes place, but when, as in pneumococci or streptococci, there is great resistance to lysis, sensitization may lead to delayed lysis anticipated by leukocytic accumulation, phagocytosis, and intracellular digestion.

It by no means follows from mere lack of parallelism, therefore, that the two serum functions are dependent upon separate antibodies.

Another important argument advanced against the identification of bacteriotropins with the bactericidal sensitizers or amboceptors is the fact that the former lead to phagocytosis without the participation of alexin, whereas the latter become active for lysis only when alexin is present.

This point also has constituted Neufeld's strongest support for maintaining that the bacteriotropins or immune opsonins are entirely distinct from the normal opsonins. It is true, indeed, that immune serum, unlike normal serum, may opsonize powerfully even after heating to temperatures which destroy alexin.

If we regard the heat-stable lytic antibody as an amboceptor in the strict sense of Ehrlich, as a specific "Zwischenkörper" with a complementophile group, this argument would have considerable weight. Even in this case, however, strong sensitization of the bacteria may make them amenable to the living cells—the phagocytes—which in itself may furnish a slight amount of alexin or alexin-like substances.

We may regard the action of the immune serum upon the antigen as rather a sensitization in the sense of Bordet, and it does not seem

[46] Neufeld and Töpfer. *Centralbl. f. Bakt.*, 1, 38, 1905.
[47] Hektoen. *Jour. Inf. Dis.*, 6, 1909.
[48] Wassermann. *Deutsche med. Woch.*, Vol. 33, No. 47, 1907.

logical to assume that the heat-stable bodies, similar in other respects, are different merely because they can sensitize bacteria both to the action of an alexin and to that of a living cell, which in itself surely contains a number of different enzymes, comparable functionally to alexin, though possibly not identical with it.

Indeed, the experiments of Dean have given much positive evidence in favor of regarding the immune opsonins or bacteriotropins as true amboceptors or sensitizers. Dean [49] found that, although heated immune serum may unquestionably opsonize by itself, its action may be still further enhanced by the addition of a little diluted normal serum (compare these results with those of Cowie and Chapin on normal opsonins). Hektoen's [50] experiments with hemopsonic immune sera are analogous. We cite one of these as illustrating the point in question:

TABLE I

Phagocytosis of Goat Corpuscles under the Influence of Goat-blood-immune Rabbit Serum, and Normal Guinea Pig Complement (Table from Hektoen, *loc. cit.*)

Immune serum		Normal guinea pig serum	Phagocytosis
.001		—	4.
.001	+	.01	20.
—		.01	0

Here, therefore, the diluted immune serum, but slightly cytotropic in itself, was powerfully activated by a diluted unheated normal serum, which in itself was entirely inactive.

Indeed, an experiment by Neufeld himself, with Bickel,[51] points in the same direction. They found that, when a heated specific hemolytic serum was added to the homologous cells in such small quantities that it no longer exerted cytotropic (opsonic) action, the addition of a small amount of alexin, too small to lead to hemolysis of the cells (and not by itself cytotropic or hemopsonic), caused active phagocytosis. Analogous experiments upon bacterial antisera were carried out by Levaditi and Inmann. It thus appears that, even in the case of the immune opsonins or bacteriotropins, we may think of a participation of two substances—a sensitizer-like one and one comparable to alexin or complement. We may, at least, infer that the full opsonic action both of normal and immune sera is dependent upon the coöperation of two such bodies. It is likely, therefore, that the mechanism of normal and of immune opsonic action may, after all, differ only in quantitative relations between the two.

For assuming this to be an antibody-alexin mechanism like hemol-

[49] Dean. *Proc. Roy. Soc. B.*, 79, 1907.
[50] Hektoen. *Jour. Inf. Dis.*, Vol. 6, 1909, p. 67.
[51] Neufeld and Bickel. *Arb. a. d. kais. Gesundh. Amt.*, Vol. 27, 1907.

ysis, we may recall the work of Morgenroth and Sachs on the relations between amboceptor and complement in hemolysis. There we saw that a large amount of amboceptor would cause hemolysis in the presence of a small amount of complement and vice versa. Therefore, here, too, in normal serum the small quantity of amboceptor or specific thermostable opsonin (bacteriotropin) may act very powerfully in the presence of the alexin. When the latter is destroyed, however, the minute quantity of specific thermostable opsonin is hardly enough to do more than initiate slight phagocytosis of comparatively non-resistant bacteria, whereas the large amount of specific sensitizer left in immune sera after inactivation may still lead to strong bacteriotropic action. In outlining this explanation we have consistently drawn upon the analogy between thermostable opsonin and amboceptor or sensitizer. Whether or not these two substances are identical is by no means positively determined and must be considered an open question for the present. However, from the above, it seems to us that much testifies in favor of such an identification.[52]

The preceding discussions have ignored the possibility that apart from opsonic or bacteriotropic action on the bacteria there may be a difference in phagocytic energy which depends upon inherent properties of the leukocyte itself.

Indeed, the technique by which the researches of Wright and his followers were carried out does not in any way take into account the source of the leukocytes as a possible variable factor. There is, however, a considerable amount of evidence which points to differences in phagocytic powers residing in the leukocytes themselves independent of the serum. Park and Biggs [53] have claimed such differences for the leukocytes of normal persons in the phagocytosis of staphylococci, and more extensive researches have been made with similar results, in the case both of staphylococci and tubercle bacilli by Glynn and Cox.[54]

The last-named authors, moreover, recognized the necessity, in making such investigations, of experimenting with leukocyte emulsions containing approximately the same number of cells, for, as Fleming [55] had shown, if unequal leukocytic emulsions are used, less phagocytosis per cell occurs in the emulsion containing the greater number of leukocytes. This phase of the subject has been taken up most thoroughly by Hektoen [56] and his associates, and Rose-

[52] Pfeiffer (quoted from P. Th. Müller) regards opsonic action as due to a combined action of amboceptor and complement and speaks of it as an "Andauung" of the bacteria for the leukocyte—which we may translate best as a partial predigestion.

[53] Park and Biggs. *Jour. Med. Res.*, Vol. 17, 1907.

[54] Glynn and Cox. *Jour. Path. and Bact.*, 14, 1910.

[55] Fleming. *Practitioner*, London, Vol. 80, 1908.

[56] Hektoen. *Jour. A. M. A.*, Vol. 57, No. 20, 1911.

now[57] has made careful comparative studies on pneumococcus phagocytosis, in which he standardized the leukocytic suspensions by actual cell counts. His work as well as that of Tunnicliff,[58] of the same school, has shown definitely that the inherent phagocytic power of leukocytes may vary not only in health and disease, but differences may exist between the cells of apparently normal people. Tunnicliff showed, for instance, that at birth the leukocytes are less active than in adult life.

For accurate experimental work, therefore, as well as in theoretical reasoning upon problems of phagocytosis, it is necessary to bear in mind the possible inherent variations in the leukocytes themselves.

Of the three factors concerned in the process of phagocytosis, then, we have considered two, the serum and the leukocytes. The former we have seen exerts a powerful determinative influence on the process, the latter a less marked influence, though still definite and measurable. We have still to discuss the bacteria themselves as variable factors in determining the degree to which phagocytosis may take place.

This problem was first investigated by Denys and Marchand in connection with their work upon streptococcus immunity, and was further studied in detail by Marchand. Marchand[59] showed that leukocytes would readily take up non-virulent streptococci in the presence of normal serum, but that under similar conditions virulent streptococci were not phagocyted at all or to a very slight degree only. He determined further that this resistance to phagocytosis remained unchanged after the virulent organisms had been killed by heat, and washed clean of culture fluid. It seemed, therefore, that the resistance depended upon a condition of the bacterial body and not upon substances secreted and given off to the environment. These experiments, as well as similar work by Mennes,[60] Gruber and Futaki,[61] and others make it clear that differences in virulence between different species of bacteria, as well as between different strains of the same micro-organism, depend, at least in part, upon the resistance which the bacterial bodies oppose to ingestion by the leukocytes. We must distinguish clearly here between these apparently purely "antiopsonic" bacterial properties and those supposedly "antichemotactic" substances which are conceived as a cause for virulence by Deutsch and Feistmantel[62] and by Bail[63] in his so-called "aggressins." The latter are supposed to be secreted bacterial

[57] Rosenow. *Jour. Inf. Dis.*, 7, 1910.
[58] Tunnicliff. *Jour. Inf. Dis.*, 8, 1911.
[59] Marchand. *Arch. de Méd. Exp.*, No. 2, 1898.
[60] Mennes. *Zeitschr. f. Hyg.*, Vol. 25, 1897.
[61] Gruber and Futaki. *Münch. med. Woch.*, 1906.
[62] Deutsch and Feistmantel. Quoted from Sauerbeck. *Lubarsch und Ostertag*, Vol. 2, 1906.
[63] Bail. *Arch. f. Hyg.*, Vol. 52, 1905.

substances by means of which the leukocytes are held at bay. The properties we are, at present, considering are probably in no way antichemotactic, but oppose purely the actual ingestion by the leukocyte, nor do they seem to depend upon the secretion of substances which injure the leukocytes. For, in the first place, a profuse accumulation of leukocytes may follow the injection of virulent micro-organisms, and Denys (quoting from Gruber) has seen active phagocytosis of virulent pneumococci, but none of virulent streptococci when antipneumococcus serum was injected with the mixture.

Rosenow [64] has carried out a thorough investigation dealing with these relations in pneumococcus infection. Seventy-five strains of this organism were all found non-phagocytable when first isolated and the resistant condition was associated with virulence for rabbits and guinea pigs. It was found, moreover, that the resistance to phagocytosis was dependent upon the inability to absorb opsonin. For, while phagocytable non-virulent pneumococci absorbed specific opsonin from serum, the virulent ones failed to do this in proportion to the degree of their virulence. Furthermore, extraction of the bodies of the virulent organisms in NaCl solution yielded a substance which inhibited the action of pneumococcus opsonin—a true antiopsonin—which he speaks of as "virulin." This discovery, if confirmed, would supply us with a very simple explanation for some phases of the problem of virulence. It is, indeed, likely that the antiopsonic property is closely bound up with chemical and structural changes which take place in the bacterial cell as it adapts itself to the parasitic conditions. This is plain from the fact that pneumococci and some other bacteria will rapidly lose their virulence when cultivated on artificial media devoid of animal serum, will retain it longer if grown on some serum media, and will rapidly regain it if passed through animals. The formation of a capsule is unquestionably a morphological evidence of such a change. Habitually capsulated bacteria, like the Friedlander bacillus, and *Streptococcus mucosus,* are of fairly constant virulence, while in other micro-organisms like the pneumococci, anthrax bacillus, plague bacillus, and certain other streptococci, the formation of a capsule goes hand in hand with an increase of virulence. By the aid of this morphological earmark of virulence, moreover, Gruber and Futaki have obtained further proof that the resistance to phagocytosis in these cases is due to the nature of the bacterial cell body rather than to any secreted antiopsonic substances. For, after the injection of anthrax bacilli into guinea pigs, they saw that leukocytes would take up uncapsulated bacilli, apparently picking them out of the midst of surrounding capsulated organisms which they were unable to ingest.

[64] Rosenow. *Jour. Inf. Dis.,* Vol. 4, 1907.

THE OPSONIC INDEX

Wright's [65] investigations upon phagocytosis were, indirectly, the outcome of his earlier work upon antityphoid vaccination. His purpose in these studies had been a purely practical one, and he had attempted to obtain a guide for the dosage and the interval between injections by measuring the bactericidal and agglutinating powers of the blood serum. In the case of typhoid immunization this was indeed a practicable method of control, since the bactericidal power of the blood serum rose directly as the immunization of the patient was attained. In the cases of many other bacteria, however, this method of study was not practicable, and Wright, as others before him, did not find a regularly increased specific bactericidal power in the blood sera of immunized animals or of patients convalescing from infections with such bacteria as the staphylococcus, streptococcus, *Micrococcus melitensis,* the *Bacillus pestis,* and a number of others. In fact, together with Windsor,[66] he showed that normal human blood has practically no bactericidal power for pyogenic staphylococci and that antistaphylococcus inoculations or recovery from an infection do not result in the production of such properties in the serum. These determinations are practically identical with Nuttall's [67] earlier studies on the same bacteria and, indeed, correspond with the data obtained by Metchnikoff and his followers in their work on anthrax infection. For, in discussing these investigations, we saw that very often the serum of a comparatively resistant animal is less potently bactericidal than that of a more susceptible one. We need only recall the difference between rabbits and dogs in this respect. The serum of the former is more strongly bactericidal than that of the latter, and yet rabbits are the far more susceptible animals. These relations have been studied with great care, also, by Petterson.[68] It was logical in such cases to look for the cause of resistance in the activity of the phagocytes, and this, we have seen, Metchnikoff did successfully in a large series of cases, both as regards natural and acquired immunity.

Yet the controversy between the strictly humoral and the cellular schools was by no means regarded as closed, especially since, in such cases as typhoid infection, the parallelism between increased resistance and extracellular bactericidal power was so plainly evident, while in this disease particularly (for technical reasons which will become clear as we proceed) no such parallelism with phagocytosis could at first be shown. It was because of such apparent confusion

[65] Wright. *Lancet,* 1902; *Practitioner,* Vol. 72, 1904.

[66] Wright and Windsor. *Jour. Hyg.,* Vol. 2, 1902; and Wright, *Lancet,* 1900 and 1901.

[67] Nuttall. *Zeitschr. f. Hyg.,* Vol. 4, 1888.

[68] Petterson. *Centralbl. f. Bakt.,* Vol. 39.

that Leishmann [69] undertook to study again the relation of phagocytosis to active immunity, chiefly upon staphylococcus cases that were being "vaccinated" therapeutically by Wright himself.

In order to obtain a numerical measure of the degree of phagocytosis, he developed a simple technique which, though crude, served to give him the information he sought. It consisted in taking small quantities of the blood of patients and mixing these in capillary pipettes with equal volumes of bacteria suspended in salt solution and thus incubating them.

The mixtures were then placed on slides, covered with a cover-slip, and incubated at 37° C. for varying periods. At the end of incubation the preparations were smeared upon slides and stained by Leishmann's modification of the Romanowski method, the number of bacteria in a large series of leukocytes counted and an average taken.

This method had many serious flaws, chief among them being the liability to coagulation of the preparations and the fact that, in each test, the fluid constituents as well as phagocytes, both of them variable factors, came from the same individual. While, therefore, it was possible to estimate an increase or decrease of general phagocytic power, it was impossible to analyze this in reference to its dependence either upon the condition of the cells, on the one hand, or that of the plasma or serum, on the other. Moreover, the relation of the number of leukocytes to that of bacteria in individual tests necessarily differed, and this, we have seen, adds a variable factor which renders it impossible to compare any two experiments with accuracy.

In spite of these difficulties, however, Leishmann succeeded in establishing, in a number of cases of staphylococcus infection, that an increased resistance was accompanied by an increased energy of phagocytosis.

Leishmann, however, went no further than this, and interpreted his results on the basis of the "stimulin" theory of Metchnikoff.

The subsequent studies of Wright, which began at the point at which Leishmann stopped, have been described in the preceding chapter and had, as their main result, we have seen, the discovery of the opsonins and the final confirmation of Denys' conception of the true mechanism of coöperation between serum and leukocytes in phagocytosis. In order to carry out these studies the technique of Leishmann was quite inadequate, and Wright's first task was to modify it in such a way that reasonably accurate comparative estimates of phagocytosis could be made.

It is necessary to outline Wright's method briefly in this place in order that we may consider possible sources of error and obtain

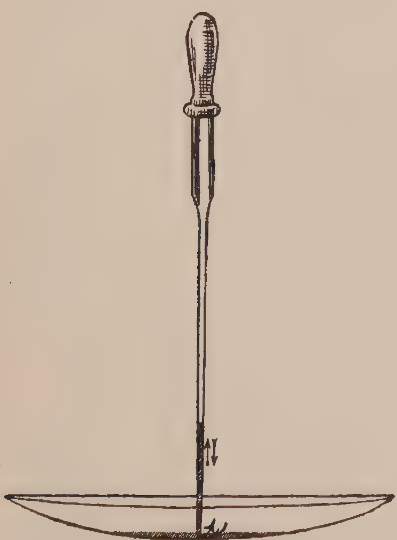
[69] Leishmann. *Brit. Med. Jour.*, 1, 1902; *Trans. London Path. Soc.*, Vol. 56, 1905.

a clear understanding of the conclusions he based on his observations.

Wright recognized that the determination of the degree of phagocytosis, induced by the opsonin of any given serum in a single test, is by itself of no value, since the actual number of bacteria taken up by each leukocyte, apart from the opsonic contents of the serum, depends also upon such purely technical factors as the concentration of the bacterial emulsion, the relative number of leukocytes, and the length of time of incubation. Two individual tests, therefore, carried out with the serum of the same patient at the same or at different times, with different bacterial emulsions or leukocytes in each, would give variable results, even though the opsonin contents themselves were entirely alike.

Wright Capsule for Taking Blood to Obtain Serum for Opsonic Tests.

In order, therefore, to obtain a relative estimate of the opsonic contents of any serum it is necessary to compare the phagocytic activity induced by this serum with the similar power of another supposedly normal serum, both tests being carried out, under exactly similar conditions, with the same bacterial emulsion and the same leukocytes. The average number of bacteria found in each leukocyte in each one of the preparations is then the "phagocytic index." The relation of the phagocytic index of the unknown serum to that of the supposedly normal serum constitutes what Wright has called the "opsonic index."



Method of Producing an Even Emulsion of Bacteria for Opsonin Determination.

Instead of using the whole blood of the patient Wright takes a small amount of blood in glass capsules, allows it to clot, and uses the expressed serum in his test. For comparison with this he employs a "pool" of a number of specimens of serum from supposedly normal individuals. By the use of such a serum mixture any slight possible variations from the normal in any one of the sera are likely to be equalized, and a closer approach to a normal standard is attained.

The leukocytes used in both tests are the same and taken, as a rule, from the blood of the worker or from some other supposedly healthy person. They are obtained by taking 15 or 20 drops of blood from the finger or ear into 5 to 10 c. c. of sodium citrate solution, in which the blood does not clot. Brief centrifugalization throws down the blood cells, with a thin, buffy coat of leukocytes on top, and these are gently taken off with a pipette. This constitutes the leukocytic cream of Wright's experiments, and furnishes a uniform leukocyte factor for the two tests which are to be compared. The bacteria are obtained by emulsifying carefully in salt solution. It is very important to obtain an emulsion free from clumps and neither

Method of Taking Up Equal Volumes of Leukocytes, Blood Serum and Bacterial Emulsion in Wright's Technique for Opsonic-index Determination.

too thick nor too thin, a result which can be secured only by experience.

Equal quantities of serum (unknown and normal "pool" respectively) are mixed with equal quantities of the bacterial emulsion and the leukocytes in capillary pipettes, and the mixtures are incubated for fifteen to thirty minutes under exactly similar conditions. At the end of this time smears are made upon slides, the preparations stained, and the numbers of bacteria in a hundred or more leukocytes counted in each of the two experiments. The average is taken, and from the *phagocytic indices* thus obtained the *opsonic index* is calculated. For instance, if

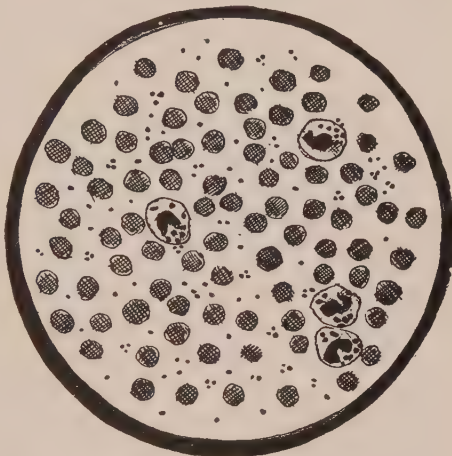
Phagocytic index (normal pool) = 8
Phagocytic index (patient's serum) = 6

then the *opsonic index* (patient's serum) = 0.75. Or, if the phagocytic index of the normal pool had been 10. and that of the patient's serum 15., then the *opsonic index* of the patient's serum, higher than normal, would be 1.5.

For the insurance of accuracy in carrying out this method Wright calls especial attention to the caliber of the capillary pipettes that are used, the concentration of the sodium citrate solution, which should be 1.5 per cent., and the freshness of the leukocytes. But it is still necessary to remember that with the greatest care in technique uncontrollable sources of error influence this method. Most important among them are the differences necessarily existing between different normal sera used for comparison and differences

in the agglutinative powers of the sera used in the two specimens. For it is plain that different degrees of agglutination may bring about great variations in the number of bacteria with which the individual leukocyte comes into contact.

Wright's method has also been particularly unsatisfactory in taking the opsonic index against such bacteria as the typhoid bacillus and the cholera spirillum, organisms which are very rapidly digested after being taken up by the leukocytes. In consequence, even after as short an incubation time as five or ten minutes, the ingested bacteria are partly disintegrated, are stained indistinctly, and cannot be counted with accuracy. In order to avoid this source of error Klien [70] has devised a modification which depends upon gradual dilution of the serum in a series of phagocytic tests with the same leukocytic and bacterial emulsions. In this way he determines the degree of dilution of the serum to be tested at which phagocytosis no longer exceeds that taking place in salt solution alone. The degree of dilution at which this result was obtained has been called by Simon the "coefficient of extinction." A comparison of sera with regard to this value, it is clear, furnished an estimate of their quantitative opsonic properties quite as instructive as the direct estimations by the Wright method, and in our opinion, at least, more reliable. Though also subject to some of the objections advanced against the Wright method, it has the definite advantages mentioned above, and is not so closely dependent upon irregularities in counting, agglutinin influences, and differences in relative proportions of bacteria and leukocytes employed. Jobling [71] has used this method with success for the standardization of antimeningitis serum.



Leukocytes Containing Bacteria. Drawing of Field as Seen in Wright's Method of Opsonic-index Estimation.

A further modification suggested by Simon, Lamar, and Bispham [72] depends upon a combination of the dilution method and a

[70] Klien. *Johns Hopkins Hosp. Bull.*, Vol. 18, 1907.

[71] Jobling. "Studies from the Rockefeller Inst.," Vol. 10, 1910, p. 614.

[72] Simon and Lamar. *Johns Hopkins Hosp. Bull.*, Vol. 17, 1906; Simon, Lamar, and Bispham, *Jour. Exp. Med.*, Vol. 8, 1906; Simon, *Jour. A. M. A.*, Vol. 48, 1907, p. 139.

modification in the method of counting. They make comparative tests of the same serum, diluted from 1 to ten to 1 to one hundred in salt solution, and estimate the opsonic power, not by determining the average number of bacteria to the leukocyte, but by taking a percentage of the total number of leukocytes which take part in the phagocytosis, that is, contain any leukocytes at all. The bacterial emulsion for this method should be so thin that, in normal serum, only about 50 per cent. of the leukocytes will contain bacteria.

That Wright's method, or any of the others, gives absolutely accurate results will hardly be claimed by any one who has worked upon opsonic-index estimations. There are certain uncontrollable variable factors, some of which have been pointed out above; and, apart from these, the delicacy of the technique is such that reliable results can ordinarily be obtained only by trained workers after considerable practice and experience. Even in such hands the percentage of personal error is more likely to be above than below 10 per cent. For ordinary clinical purposes, therefore, in the control of cases the estimation of the opsonic index is not often practicable.

On the other hand, there can be little doubt about the fact that careful comparative estimation, by Wright's method and by some of the modifications, carried out by workers with experimental training and consequent attention to extensive controls, have yielded results of sufficient accuracy to permit the recognition of definite facts concerning opsonins. It is beyond question, therefore, that the conclusion regarding the relation of opsonic fluctuations to clinical conditions and the general significance of opsonins emanating from laboratories like those of Wright, Neufeld, Hektoen, and some others may be accepted as fact—especially since in most essentials such workers have agreed. In consequence we are now in possession of knowledge regarding the opsonic constituents of the blood in health and disease, and in the course of active immunization with bacterial vaccines, which is of the greatest practical importance. We may summarize the results of such investigations by saying that in many of the infections of man the resistance of the patient is roughly proportionate to the opsonic index—and that properly spaced inoculation with suitable quantities of dead bacteria (vaccines) will raise the opsonic index and lead to recovery in many of the localized subacute and chronic conditions.

Relation of Opsonic Index to Clinical Condition.—As to the usefulness of the treatment in various infections and the limitations within which we may hope for results opinions differ, and these will be discussed more fully below. Before we proceed to this, however, it will be useful to consider the studies upon which the parallelism between opsonic index and clinical condition was founded.

Wright's own earlier studies were made chiefly upon staphylococcus infections and tuberculosis. Since then the method has been

applied to almost all known infections with varyingly successful results.

One of the first steps in determining such a parallelism between the resistance of a patient and the opsonic index consisted, of course, in comparing the index of the sera of normal individuals with that of patients suffering from infection. Wright and Douglas did this in a large series of studies. In the case of staphylococcus infections the following experiment will illustrate their results:

TABLE I

(Wright and Douglas, *Proc. Royal Soc.*, Vol. 74, 1904.)

Showing the ratio in which the phagocytic or opsonic power of the patient's blood stood in each case to the phagocytic or opsonic power of the normal individual who furnished the control blood. (The phagocytic power of the control blood is taken in each case as unity.)

Initials of Patient	Form of Staphylococcus Invasion	Opsonic Index
E. E.	Furunculosis	0.48
F. F.	Sycosis	0.49
J. E.	Acne	0.64
J. H.	Furunculosis	0.87
W. B.	Acne	0.55
E. H.	Acne	0.82
W. H.	Furunculosis	0.79
R. G.	Furunculosis	0.7
G. L.	Acne and sycosis	0.74
S. C.	Furunculosis	0.87
W. L.	Furunculosis	0.88
W. P.	Furunculosis	0.39
S. F.	Very aggravated sycosis	0.1
E. F. D.	Acne	0.73
D. C.	Sycosis	0.8
J. M.	Acne	0.48
W. M.	Sycosis	0.37
E. P.	Acne	0.6
M. S.	Pustular affection of lips	0.6
F. V.	Repeated staph. infection	0.47

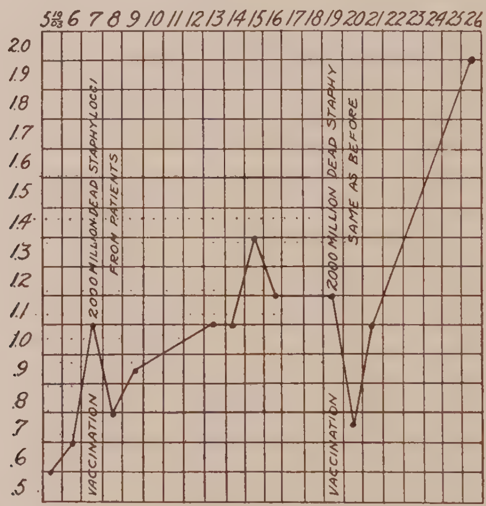
In this series, as in others investigated by Wright and his collaborators, staphylococcus infection was uniformly associated with a low index. He concludes that there is probably a causative relation between the two facts, in that under conditions of depressed phagocytic powers staphylococci may gain a foothold, while under ordinary normal conditions they would fall prey to phagocytic destruction soon after entering the body.

The study of the opsonic index during the treatment of such cases with dead staphylococcus cultures (usually with the organisms cultivated from the patient's own lesions—"autogenous vaccines")

revealed a striking coincidence between the rise of the opsonic index and improvement in the clinical conditions. A number of further interesting and practically important points were brought out by the systematic study of these relations which may be illustrated by reproducing a plan of the opsonic index curves constructed from cases.

The curve shown above, and taken from a paper by Wright and Douglas, illustrates the course of the opsonic fluctuations in the case of a medical student who had suffered for four years from boils.

When first seen the opsonic index (1. being normal) was 0.6,



CURVE I.—RESULT UPON OPSONIC INDEX OF VACCINE TREATMENT IN TWO CASES OF CHRONIC STAPHYLOCOCCUS FURUNCULOSIS.

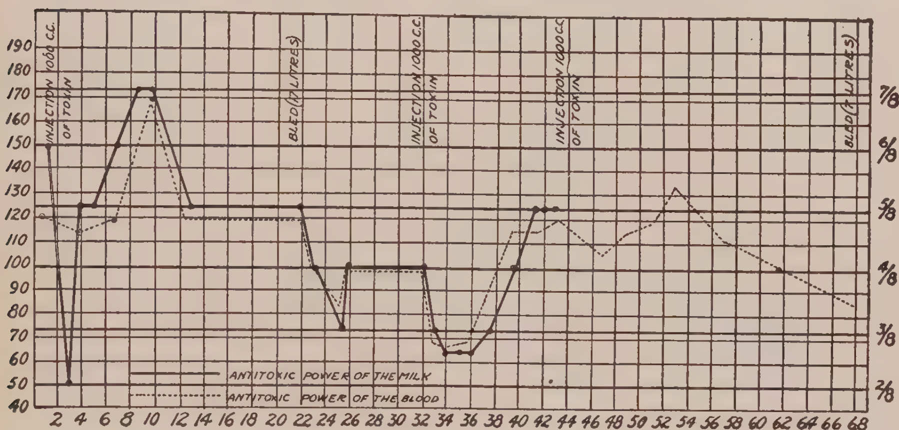
(After Wright and Douglas, *Proc. Royal Soc.*, Vol. 74, 1904, p. 156; also from "Studies on Immunity," p. 41.)

and there were 2 boils on the neck. For 3 days after this there was a spontaneous rise in the index accompanied by an improvement of the lesions.

On the third day 2 billion staphylococci were injected. This was followed by an immediate drop of the phagocytic power—(*the negative phase*); together with this a new boil began to form. Soon, however, the opsonic power began again to rise, this time considerably above normal, reaching its highest point on the 8th day, when it again began to diminish. A second inoculation on the 12th day was followed by a similar preliminary negative phase, then a steady and rapid positive phase, which was accompanied by cure.

The rise and fall of the opsonins after the injection of bacteria is entirely analogous to the similar fluctuations of other antibodies

after antigen injections. Measurements of this kind are numerous in the literature. Thus Salomonsen and Madsen, measuring the antitoxin contents of the blood and milk of a mare which were being immunized by injections of diphtheria toxin, obtained the following curve, which is entirely similar in essential features to those constructed for the opsonic index by Wright and Douglas:



Curve Describing Quantitative Measurements of Antitoxin in a Mare in Response to Toxin Injections.

(Taken from article by Salomonsen and Madsen, *Ann. de l'Inst. Pasteur*, Vol. 11, 1897, p. 319.)

Results having the same general significance are apparent in the measurements made upon a tetanus toxin goat by Ehrlich and Brieger,[73] and in the observations upon the fluctuations of bactericidal power of the sera of patients treated with typhoid vaccines made by Wright[74] himself. Similar, again, are the various agglutinin curves constructed by Jorgensen and Madsen[75] and others.

Apart from the purely theoretical value of such measurements, they demonstrate features which are therapeutically of the greatest importance. They show that in all processes of active immunization the injection of antigen is followed almost immediately by a rapid decline of specific antibodies in the blood serum. This "negative" phase, as it is called, is possibly due to a neutralization of existing antibodies and lasts for varying periods, which must, of course, depend upon complex relations between the degree of resistance (or amount of antibody constituents of the serum), the quantity of antigen injected, and the general recuperative powers of the subject.

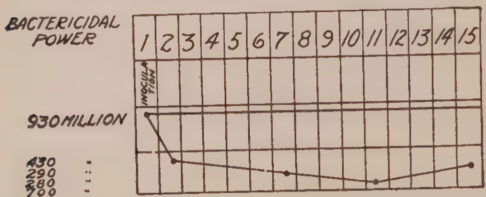
[73] Ehrlich and Brieger. *Zeitschr. f. Hyg.*, Vol. 13.

[74] Wright. *Practitioner*, Vol. 72, 1904, p. 118.

[75] Jorgensen and Madsen. *Festschrift*. Serum Institut. Kopenhagen, 1902.

Therefore, without some control like that furnished by the measurement of opsonins or other antibodies it is impossible to determine whether the negative phase has ended or is still in progress unless the clinical condition is of such a nature or location that degrees of improvement or exacerbation are well marked and easily observed. Even then clinical observation alone is at best not an absolutely reliable guide.

The practical importance of the question lies in the harm which may accrue to the patient if a second injection is practiced before the cessation of the negative phase. Wright himself accentuates this danger by expressing the opinion that, in typhoid inoculations, an excessive dose administered to a patient in the physiological condition of the negative phase may be followed by a prolongation of this phase into a period of several months.



Prolongation of the Negative Phase Due to Too Vigorous Treatment with Typhoid Vaccine.

(After A. E. Wright, *Brit. Med. Jour.*, May 9, 1903. Also from "Studies on Immunity," p. 179.)

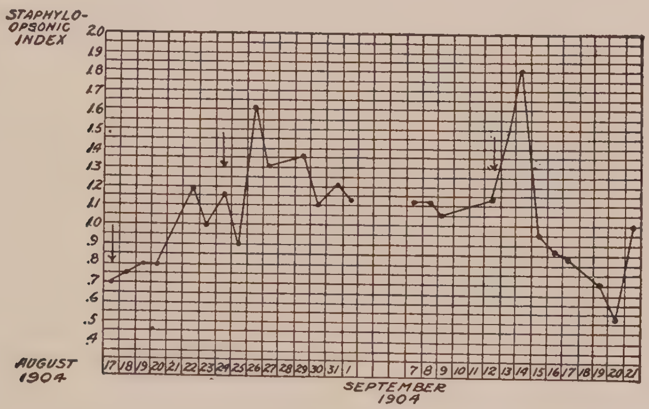
In the case of successive inoculations, as in vaccine treatment, a too rapid repetition—i. e., a repetition of injection during such a period of depression—leads to what Wright speaks of as a "summation of the negative phase," which obviously may seriously aggravate the condition of the case.

It is to such a cumulation of the negative phase that Wright attributes the failures attendant upon the use of tuberculin during the early days after its introduction, since injections at this time were carried out without any control of serum reactions in the patient and with comparatively large doses.

The danger to be carefully avoided, therefore, is a too rapid succession of inoculations and too large a dosage, since both of these procedures may be followed by cumulation of the "ebb tide of immunity," and great harm may result. On the other hand, if the treatment is so spaced and measured that the successive inoculations are given just before the positive phase has ended—in other words, just before the apex of the curve is reached—a moderate negative phase may be then followed by a second positive phase still higher than the first, and corresponding improvement will result. It is even possible to occasionally obtain a summation or cumulation of the positive phase—in which the negative phase will be entirely suppressed. This is illustrated in the following curve, in the case of

the first and second inoculation indicated on the chart. This case, too, was a staphylococcus infection occurring in a laboratory attendant:

Such a summation of positive phase, though of course the ideal to be aimed at, cannot be produced with regularity, however carefully we may attempt to control the treatment. It is worth mentioning, moreover, a fact which should become evident from the preceding and is too often overlooked, that a summation of the negative phase can certainly be attained by the frequent repetition of larger doses.



STAPHYLOCOCCUS INDEX AS DETERMINED BY WRIGHT IN A CASE OF ACNE TREATED WITH STAPHYLOCOCCUS VACCINES.

Note summation of positive phase after third injection. (After A. E. Wright, "Studies on Immunity," p. 348.)

This is practiced not infrequently in the false hope of hastening the acquisition of immunity, and does harm more often than good.

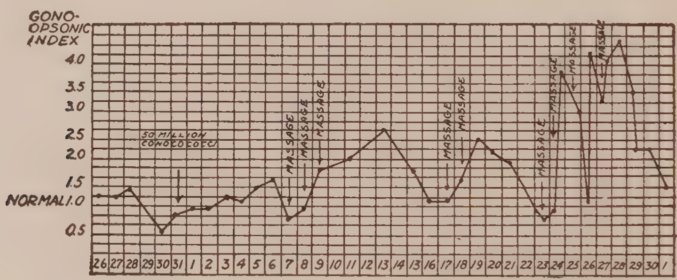
Ordinarily the opsonic index when raised to a level considerably above normal will gradually recede to the normal or even to a subnormal condition. In isolated cases, however, especially in tuberculosis, the index may remain high for periods as long as a month. This Wright speaks of as a sustained "high tide" of immunity. These laws of fluctuation are all of them entirely analogous to those long well known in the cases of other antibodies, for even in diseases in which the immunity following an attack—(typhoid fever, cholera, plague, and others)—is continued through life the antibodies disappear from the blood after varying periods and we are forced to seek the cause of the permanently high resistance, not in the circulating blood, but in the ultimate physiological units—the cells and tissues.

According to Wright also, the treatment with vaccines may be either reënforced or entirely replaced by a process of autoinoculation from the patient's own lesion by increasing the local cir-

culation, thereby throwing more of the specific antigen into the blood stream.

This reasoning has been applied, not only to the treatment of tuberculosis and other conditions, but has been utilized to explain fluctuations in the opsonic indices of untreated patients under the influence of unusual motion of the diseased parts—as in walking or other exercise. Wright's meaning is well illustrated by the following curve of opsonins in a case of gonorrheal polyarthritis in which massage of the joints resulted in reactions similar to those ordinarily elicited by vaccine injections:

A further modification of the vaccine treatment of Wright originated in the observation that the exudate present in many infected



Opsonic Curve in a Case of Gonorrheal Arthritis in Which Auto-Inoculation by Massage Was Practiced.
(After Wright, Douglas, Freeman, Wells and Fleming, "Studies on Immunity," p. 373.)

foci is often very much less rich in opsonins than is the blood serum of the same patient. This is not unlikely to be due to an absorption of the antibodies by the bacteria—as well as by the tissue detritus in the lesion. But Wright has interpreted it as a purely specific absorption by the bacteria, and has utilized it for diagnostic purposes. Thus, with Reid,[76] he has examined in this way the comparative amounts of tubercle bacillus-opsonins in the blood, and in the local exudates (peritoneal fluid) in cases suspected of tuberculosis, and has determined the tuberculous nature of the condition by showing a discrepancy between the two. These results have not been universally confirmed.[77] But therapeutically, because of this supposed lack of opsonin in the fluid of lesions, Wright has advised the increase of the local flow of lymph by poulticing, heat, drainage, Bier's cups, X-rays, Finsen light, and other means of accomplishing this purpose.

All that has gone before (most of it taken directly from the

[76] Wright and Reid. *Lancet*, 1906; *Proc. Roy. Soc.*, Vol. 77, 1906.
[77] Opie. *Assoc. of Am. Phys.*, Washington, 1907.

staphylococcus studies of Wright and his immediate followers) has tended to show a very close correspondence of clinical improvement with the increased opsonin contents of the blood.

As applied to other infections, such as gonococcus arthritis, colon bacillus cystitis, localized pneumococcus lesions, and many other conditions of a localized character, observations of a similar general significance have been made. Such reports have been made, apart from the Wright school, by Emery,[78] Potter, Ditman, and Bradley,[79] Potter,[80] Tunnicliff,[81] Whitfield,[82] Cole and Meakins,[83] and many others, and we may say with reasonable accuracy that, in localized infections particularly, there is much evidence to show that clinical improvement and rise of the opsonic index go hand in hand.

There have been many exceptions to this—which, in view of the complicated factors involved in immunization, as well as the difficulty of the technique, is not surprising.

In tuberculosis—in which many of Wright's earlier studies were made—the parallelism has not been so consistent. Thus even the early work of Bullock [84] showed that, in contrast to similar staphylococcus investigations, the tuberculo-opsonic indices of patients may occasionally be higher than normal, and similar observations were made by Lawson and Stewart [85] in cases of acute pulmonary tuberculosis.

However we analyze the work done on tuberculo-opsonins—and the investigations on this subject are far too numerous to be here reviewed—we are forced to the conclusion that in this disease the opsonic fluctuations are far more irregular than in most other conditions. Much,[86] for instance, found no regular differences between the tubercle bacillus opsonins of healthy and of diseased individuals, and Koehlisch [87] obtained similar results, adding the important observations that animals that show a high natural resistance to the human type of the tubercle bacillus invariously show an opsonic index much lower than that of man.

We may question with much justice, therefore, whether in the case of this bacillus opsonic investigations can be looked upon as indicators of immunity with as much confidence as in cases of other bacterial invasions. It is true, indeed, that tubercle bacilli—as well as leprosy, rat leprosy, and other acid-fast bacteria—are eagerly

[78] Emery. "Immunity, etc.," Lewis, London, 1909.

[79] Potter, Ditman, and Bradley. *Jour. A. M. A.*, Vol. 47, 1906, p. 1722.

[80] Potter. *Jour. A. M. A.*, Vol. 49, 1907, p. 1815.

[81] Tunnicliff. *Jour. Inf. Dis.*, Vols. 4 and 5, 1907 and 1908.

[82] Whitfield. *Practitioner*, May, 1908.

[83] Cole and Meakins. *Johns Hopkins Hosp. Bull.*, Vol. 18, 1907.

[84] Bullock. *Transact. of Lond. Path. Soc.*, Vol. 56, 1905, and *Lancet*, 1905, Vol. II, p. 1603.

[85] Lawson and Stewart. *Lancet*, 1905, Vol. II, p. 1406.

[86] Much. *Münch. med. Woch.*, p. 496, 1908.

[87] Koehlisch. *Zeitschr. f. Hyg.*, Vol. 68, 1911.

taken up by polynuclear leukocytes when they are injected into the peritoneal cavity of a guinea pig or rat or other experimental animal. On the other hand, we have much evidence which seems to show that such phagocytosis is not in these cases a direct method of bacterial destruction. In another place we have cited the experiments of Tschernorutski,[88] which showed that polynuclear leukocytes, though containing other ferments, were devoid of lipase. And Carey and the writer—experimenting with rat leprosy bacilli—found that these acid-fast bacteria were not disintegrated within leukocytes in the course of weeks, while they were often subject to rapid destruction in the presence of living spleen cells in plasma. Furthermore, in the discussion of the tuberculin tests we have reviewed evidence which points to the fact that in the reactions to tubercle bacilli we have probably to deal more particularly with sessile receptors on fixed tissue cells than with specific circulating antibodies. Bartel and Neumann [89] have concluded that the phagocyte which takes up tubercle bacilli represents only a preliminary vehicle by which the micro-organisms are conveyed to the spleen and lymphatic tissues, in which actual destruction then takes place. While no final conclusions can be drawn from the available evidence, all these data render it uncertain whether the opsonic index as determined for polynuclear phagocytosis may be at all regarded as a reliable indication of increased or diminished resistance, and on this basis the control of therapy in tuberculosis by opsonin estimations is of course placed upon an uncertain basis.

We have then very briefly traced the work done upon opsonin determinations from the purely practical point of view. There is of course no question about the scientific accuracy of the observations upon which rests our knowledge of the opsonic properties of blood serum. There is also no doubt concerning our ability to increase the immunity of an individual by systematic treatment with vaccines made of pure cultures of bacteria. However, the work of Wright has concerned itself with two distinct questions which must be separately answered. Briefly stated these are: *1.* What is the value of opsonic estimations in controlling the therapeutic vaccinations of patients? *2.* To what degree and in which particular conditions may the process of vaccination (active immunization) be regarded as a hopeful method of therapy?

The first question has, in part, been answered in the preceding paragraphs. Reasonably accurate comparative estimations of the opsonic properties of serum can unquestionably be made by Wright's method, or some of its accepted modifications, in the hands of trained workers who look upon each estimation as an experimental problem

[88] Tschernorutski. *Hoppe-Seyler's Zeitschr. f. Phys. Chem.*, Vol. 75, 1911.
[89] Bartel and Neumann. *Wien. klin. Woch.*, Nos. 43 and 44, 1907; *Centralbl. f. Bakt.*, Vol. 48, 1909.

and have time for control and repetition. That even in such cases the matter is difficult is amply testified to by such reports as that of E. C. Hort,[90] who states that two of the most skilled experts [91] in London, working with samples of the same serum taken before and after vaccination, reported—"the one that the index was raised, the other that it was lowered by the treatment." This, and similar experiments of other observers, do not, of course, invalidate the results obtained in special researches like those of Wright, Neufeld, and others, but they *do* indicate that the control of clinical cases by opsonic estimations is not a matter that can profitably be made a routine procedure by which the treatment of the cases can be regulated. As a problem of clinical research in a given series of patients opsonin studies are unquestionably valuable and the comparative data so obtained have proved, and will continue to prove, of great value. But we cannot hope as yet, it seems to us, to utilize this method, except in cases in which much time and care can be centered upon a few patients under the best conditions. Opinions essentially similar to this have been expressed by experienced clinicians (Potter,[92] for instance), who have followed out series of cases on which systematic opsonin determinations were made.

As to the opsonic index in tuberculosis, we believe that the experimental evidence at present available does not show that such measurements are reliable measures of resistance, and, in this disease, even when the index is taken with a degree of care which precludes gross error, it is doubtful whether its estimation is of as much value in controlling treatment as are the data obtained by skilled clinical observation.

This leaves us, therefore, for the control of vaccine treatment in the routine work of the clinic only the information gleaned from such indications as alterations in any visible or palpable lesions, general systemic symptoms, temperature, leukocytosis, etc. Since these will present such manifold and variable pictures in different conditions, generalization is useless.

[90] Hort. *Brit. Med. Jour.*, Feb., 1909, p. 400.
[91] Quoted from Adami, *Trans. Amer. Phys. & Surg.*, Vol. 8, 1910. See also Pearson, *Biometrica,* 1911.
[92] Potter. *Loc. cit.*

CHAPTER XVI

ANAPHYLAXIS

Historical Survey.—The fundamental principle of active immunization is the fact that the treatment of animals with bacteria or bacterial products, carried out according to certain empirically determined methods, leads to increased tolerance or resistance. The limitations within which this statement is true, and the variable factors to which it is subject, we have considered in the foregoing discussions dealing with the antibody-antigen reactions.

Although these reactions were studied at first purely from the point of view of increased resistance to infection, the most extensive studies of antibody formation have been made with such antigens as blood cells, serum, and other substances which are in themselves entirely harmless. For, in such reactions, great simplicity and ease of experimentation could be attained. For a time, therefore, the primary problem of increased tolerance or resistance was relegated to a secondary position, or, at least, dealt with chiefly by analogy, and the phenomena of increased antibody formation and increased resistance to the antigen were assumed to maintain a more or less strict parallelism.

That the problem is not as simple as this has gradually become obvious. We have come to recognize that the treatment of animals with any antigen, bacterial or otherwise, though leading to increased tolerance under certain conditions and within definite limits, may, under other conditions, give rise to the very opposite, that is, to an intolerance or increased susceptibility.

The development of this knowledge, like much else that serum study has revealed in the last fifteen years, takes root in isolated observations scattered throughout the early literature, but often regarded as merely noteworthy accidents or technical errors. This particular problem, moreover, was confused by the fact that some of the earliest observations regarding hypersusceptibility were made in the course of experimentation with diphtheria and tetanus toxins, antigenic substances toxic in themselves and, therefore, as we shall see, clouding some of the basic principles apparently involved in the phenomenon of which we now speak as anaphylaxis. We will for the present, therefore, limit our discussion to the development of the knowledge of anaphylaxis merely as it concerns the hypersusceptibility incited in animals and man by treatment with various antigens,

such as animal sera and other proteins, which possess but slight native toxicity or no toxicity whatever in themselves.

The special problem of toxin hypersusceptibility ("Giftüberempfindlichkeit" of von Behring) we will deal with later in a separate section, since it is as yet very doubtful whether these phenomena may justly be incorporated with true anaphylaxis as we now define it, despite the admitted fact that attention was called to the problems of acquired susceptibility largely because of these toxin investigations.

The earliest observation having direct bearing upon protein anaphylaxis is one which Morgenroth discovered in the writings of Magendie. Morgenroth [1] mentions that, in his "Vorlesungen über das Blut," published in 1839, Magendie describes the sudden death of dogs which had been repeatedly injected with egg albumen. Although Morgenroth, whose paper was written before the present facts regarding hypersusceptibility were fully developed, attributes these results to the action of precipitins, there can be little doubt as to the anaphylactic nature of Magendie's results.

A clear statement of the fundamental phenomena was given, also, by Flexner,[2] in 1894. In describing certain experiments he says: "Animals that had withstood one dose of dog serum would succumb to a second dose given after the lapse of some days or weeks, even when this dose was sublethal for a control animal."

One of the experiments cited to justify this statement is as follows:

"Two rabbits received ½ of 1 per cent. and 1 per cent. of their body weight respectively of dog's serum, twenty-four hours old, on January 19, 1894. With the exception of hemoglobinuria, indisposition to move, and increased respiration, no ill effects were noted. The animals still showed hemoglobinuria on the following day. These symptoms disappeared and apparently the rabbits entirely recovered. On February 12, 1894, each received 1 per cent. of their body weight of dog's serum intravenously. A control animal also received 1 per cent. of its body weight of the same serum. The two animals that had been previously inoculated died in two and twelve hours respectively; the control animal showed only hemoglobinuria which disappeared after a day or two."

The experiment here quoted is, as a matter of fact, a perfect example of what we now know as "active sensitization."

However, the isolated observations recorded above were neither correlated nor followed out to their logical developments, and a systematic and purposeful study of the problem was deferred until Richet and Portier [3] attacked it in 1902.

Richet and Héricourt [4] had observed in 1898 that dogs treated with eel serum, which is toxic *per se,* could be killed by a second injection of an amount too small to injure normal untreated animals. Some years later Richet, in collaboration with Portier,[5] determined a similar fact in the case of a poisonous substance, "actinocongestin," which they isolated by extraction of the tentacles of actinia.

[1] Morgenroth. "Ehrlich Gesammelte Arbeiten," Transl., Wiley & Son, N. Y., 1906; p. 332 footnote.

[2] Flexner. *Medical News,* Vol. 65, p. 116, 1894.

[3] Richet and Portier. *C. R. de la Soc. Biol.,* p. 170, 1902.

[4] Richet and Héricourt. *C. R. de la Soc. Biol.,* 1898.

[5] Portier and Richet. *C. R. de la Soc. Biol.,* p. 170, 1902.

Some of the facts of Richet and Héricourt's observations are as follows: Actinocongestin injected intravenously into dogs in quantities of 0.05 to 0.075 gram per kilo weight may cause illness, with vomiting, diarrhea, and respiratory distress, but does not kill. A dose of 0.002 gram per kilo causes no symptoms in a normal dog. If, however, 0.002 gram of the poison is injected into a dog which has previously received a sublethal dose and recovered, the result is violent illness and often death. It was obvious, and this was clearly stated by Richet, that the first dose had induced a condition of markedly greater susceptibility to the poison.

He, therefore, spoke of the phenomenon as "anaphylaxis" ("action anaphylactique de certains venins") to express its antithesis to prophylaxis or protective effects.

Although it has been disputed by a number of writers that Richet's investigations constitute the beginnings of our modern understanding of the anaphylactic phenomena, yet his recognition of the distinct dependence of the hypersusceptible condition upon a preceding inoculation with the same substance, and his conclusion that a definite incubation time must elapse after the first injection before susceptibility is developed, defined two of the most important criteria of the condition and initiated purposeful investigations in this field. It is true, on the other hand, that, like v. Behring and most of his other predecessors, he was working with primarily toxic substances, and the final recognition of the general biological significance of the anaphylactic phenomenon was necessarily deferred until a similar development of hypersusceptibility was noted in animals injected with various antigens which of themselves were entirely harmless. In this the history of anaphylactic investigations is similar to that of other reactions to antigen injections, lysin, agglutinin, and precipitin formation, in which the first observations were made upon pathogenic bacteria or their products, and in which subsequent extension of the investigations revealed that the response to inoculation with bacterial proteins represented merely a single phase of a general biological reaction on the part of animals to treatment with the large class of substances known as antigens.

This generalization of Richet's observations had really been foreshadowed by the observations of Magendie and by the experiments of Flexner quoted above, but this work had been lost sight of and the attention of investigators was again focused upon the problem mainly by the publication of Arthus [6] in 1903 on the repeated injection of horse serum into rabbits, and some observations made upon guinea pigs by Theobald Smith and communicated by him in 1904 to Ehrlich.

Arthus [7] found that horse serum injected into rabbits by any of the usual paths of entrance is entirely innocuous. It is possible to inject 10, 20, or even 40 c. c. without harm. If, however, one repeatedly injects small amounts, 5 c. c. or less, subcutaneously, at intervals of several days, eventually the later injections will give rise to infiltrations, edema, sterile abscesses, and even gangrene at the points of injection. He recognized that this was not due to cumulative action, and that it was not necessary to inject several times in the same place to produce the characteristic response. For instance, the early injections might be made into the peritoneum, the subsequent ones into the skin, and the local reactions to the later injections might nevertheless ensue. In other words, he recognized the systemic nature of the phenomenon and regarded it as analogous to the observations of Richet in that he spoke of the hypersensitive rabbits as "anaphylactisés" by a series of preparatory injections.

[6] Arthus. *C. R. de la Soc. Biol.*, Vol. 55, p. 817, Réunion biol., Marseille, June, 1903.

[7] Arthus et Breton. *C. R. de la Soc. Biol.*, 55, p. 1478.

The "phenomenon of Theobald Smith" is closely related to that of Arthus, and was made in the course of the standardization of diphtheria antitoxin in guinea pigs. It was noticed that guinea pigs which had been used for this purpose and had survived had acquired great susceptibility to subsequent injections of normal horse serum made several days or weeks later.

With these observations as points of departure, together with the studies of v. Pirquet and Schick [8] upon the clinical manifestations of antitoxin injections into human beings, a number of investigators took up the problem, chief among them Rosenau and Anderson, of the United States Hygienic Laboratory, and R. Otto, of the Frankfurt Institute of Experimental Therapy.

Although the paper of Otto [9] appeared in print a little earlier than did the first one of the American workers, the investigations were independent and almost synchronous. Their results, moreover, confirm each other in all essentials. Otto showed that the Theobald Smith phenomenon was entirely independent of the toxin or antitoxin contents of the injected serum, but could be produced (though somewhat less markedly) with horse serum alone. He also showed that, while a preliminary injection of horse serum "sensitized" a guinea pig to a subsequent dose given after an interval of 10 to 12 days, the repeated injection of considerable quantities at short intervals produced a condition of "antianaphylaxis" or immunity to the later injections. Otto, too, excluded from his results the direct relation of the anaphylactic state with the possible presence of serum precipitins, a thought suggested by Morgenroth in his interpretation of the observations of Magendie mentioned above.

Rosenau and Anderson [10] had attacked the problem with the primary purpose of throwing light upon the occasional accident of sudden death following the injection of diphtheria antitoxin into human beings. Since the detailed description of their extensive investigations would tend to render more difficult the exposition of an already sufficiently complicated subject, it will be best to tabulate the chief results of this classical series of their earlier papers. Briefly, these are as follows:

1. A single injection of horse serum into guinea pigs, harmless in itself, renders these animals hypersusceptible to a subsequent injection given after a definite interval or incubation time.

2. This interval, with the ordinary dosages employed (about 1 to 2 c. c.), was about 10 days. Properly carried out injections after this period were usually fatal.

3. The known antibodies, antitoxins, hemolysins, and precipitins, are not responsible for the reaction.

4. The reaction is specific, injections of horse serum sensitizing to horse serum only. (The question of specificity will be further discussed below.)

5. The sensitive condition is transmissible from mother to offspring,[11] the young of sensitized mothers being hypersusceptible to a *first* injection of horse serum.

6. The reaction is extremely delicate. Rosenau and Anderson succeeded

[8] Von Pirquet u. Schick. "Die Serumkrankheit," Deuticke, Wien, 1906.

[9] Otto. "Das Theobald Smithsche Phaenomen, etc., v. Leuthold Gedenkschrift," Vol. 1, 1905; also Otto in Ergänzungsband 2, "Kolle u. Wassermann Handbuch," etc.

[10] Rosenau and Anderson. *U. S. Pub. Health and M. H. S. Hyg. Lab. Bull.* 29, 1906; 30, 1906; 36, 1907; *Jour. Med. Res.*, Vol. 15, 1906, Vol. 16, 1907; also *Jour. Inf. Dis.*, Vol. 4, 1907, Vol. 5, 1908.

[11] It is important practically, as Anderson points out, that a female guinea pig may transmit to its young sensitiveness to horse serum and immunity to diphtheria toxin.

in sensitizing in one case with 0.000001 c. c. (one one-millionth) of horse serum.

7. The hypersusceptible state is not a transient condition, but may last a long time.

8. Sensitization, or the production of the hypersusceptible condition, can be carried out, not only with the various animal and vegetable proteins employed in the first experiment, but can be brought about by the use of extracts of various bacteria. In such cases also the reaction is specific. The first determinations with bacterial extracts carried out by Rosenau and Anderson were made with colon, anthrax, typhoid, and tubercle bacilli.

By these observations, then, the possibility of a direct relation between the phenomena of anaphylaxis and infectious diseases in animals was indicated.

This, in essence, is the harvest of the two earliest purposeful researches into this problem. A large number of investigators now took up the question, and its further elucidation, as we shall see, has proved, not only the most directly fruitful of the phases of recent immunological studies, but has thrown much indirect light upon antigen-antibody reactions apart from the anaphylactic phenomena themselves.

Varieties and Classification of Phenomena of Hypersusceptibility.—Before proceeding to considerations of detail, it may be well to make a brief survey of the many different varieties of altered reaction capacity which have been observed. It has been known for a long time that hypersensitiveness to a great many different substances in nature existed in the form of drug idiosyncracies, tuberculin reactions, hay fever, etc., etc. The relationship of these forms of increased reaction capacity to the phenomena described at a later date as "anaphylaxis," is an important and, in many cases, an as yet unsolved problem. For this reason, Doerr [12] has made a very useful contribution to the study of hypersensitiveness by an attempt at a logical classification of these occurrences. Doerr adopted the term "Allergy" which was first used by von Pirquet in his studies on serum sickness and extended its meaning to include all forms of changed reaction capacity, whether the substance to which this altered reaction was evident, was an antigen or not. Von Pirquet, then, had used the term merely in the sense of signifying altered reaction, but he implied in his work that an antigen-antibody mechanism was at the basis of Allergy in general. Advances in the knowledge of these phenomena since von Pirquet's earlier use of the term, justify the extension of the significance of the word by Doerr. Under Allergy, therefore, Doerr gathers together all phenomena of this nature. He subdivides Allergy into two main classes, on the one hand, altered reaction capacity to substances not antigenic in nature, and, on the other hand, a similar condition of the body toward sub-

[12] Doerr, Kolle & Wassermann. *Handbuch*, Second Edition, Vol. II, p. 964.

stances that are recognized as true antigens. Abbreviated somewhat, the following scheme illustrates Doerr's classification:

ALLERGY

1. *Hypersusceptibility* (and lessened susceptibility) *to non-antigenic substances.*	2. *Hypersusceptibility to antigenic substances.* (a) Substances toxic in themselves. (b) Protein antigens not primarily toxic.

We need not in this place enter into a discussion of the minor subdivisions of Doerr's scheme since these will appear as we discuss individual phenomena below. Recently, changes in this classification have been proposed by Coca.[13] Coca has brought a number of new points of view into the discussion which, whether we agree with his conclusions or not, should, in many of their aspects, be taken seriously, since they are thoughtfully formulated and are everywhere based on sound comprehension of prevailing conditions. Coca uses the word "hypersensitiveness" to include all of the conditions discussed, and subdivides them into two main classes, namely, (a) Anaphylaxis, and (b) Allergy. He limits the term "Anaphylaxis" very definitely to phenomena in which an antigen-antibody reaction has been proven, and applies the term "Allergy" to all conditions of hypersensitiveness in which this mechanism has not been shown to be the underlying cause of the symptoms. Under the second heading he classifies drug idiosyncracies, serum sickness in man (which he speaks of as serum allergy), and hay fever. He also separates from the conception of anaphylaxis, such reactions as the tuberculin reaction and toxin hypersusceptibility. It would lead us too far afield to discuss in this place the many individual points of possible difference of opinion on the validity of Coca's disagreements with Doerr's classifications. We will take up these points as we discuss individual phenomena, and will, in general, hold ourselves to the Doerr scheme which we think is reasonable and, for the time being, logical. We deplore that Coca should have given a meaning to the word "Allergy" different from the original sense in which Von Pirquet used it, since this is likely to bring a certain amount of confusion into the literature without aiding much in clarifying his own conceptions of the actual differences in mechanism.

For our own purposes we believe that the most comprehensive way of discussing these phenomena is to group together in one class, as true anaphylaxis, those manifestations of hypersensitiveness in which we know that an antigen-antibody mechanism is involved, and

[13] Coca. "Hypersensitiveness, Practice of Medicine," F. Tice, New York, 1920.

take up in separate sections, then, other occurrences, discussing in each individual case how much we know of the mechanism and to what extent we have any right to believe that the process varies fundamentally from true anaphylactic phenomena. This is more or less the same method of treatment given to the subject in a recent review by Gideon Wells who also rigidly delimits the term "anaphylaxis" to the protein phenomena referred to, incorporating in this class only antigen-antibody reactions, and deals separately with other conditions in which there is doubt or in which such reactions can be definitely ruled out, at least within our present understanding.

While we lay the chief stress for purposes of classification upon the demonstrable occurrence of an antigen-antibody union as basic to the anaphylactic phenomena, we will see that other important differences enter. Thus, the question of inheritance, the possibility of passive transfer and of desensitization, all play rôles which have been taken as fundamentally differentiating one variety of hypersusceptibility from another. Our own opinion as we hope to express it a little further along, is very definitely that many of these differences are more apparent than real, in that there are certain deep underlying processes which will bind all these phenomena of specific hypersensitiveness together. Indeed, other writers tacitly admit a similar opinion by the fact that they include all these matters under the heading of hypersusceptibility. Our scheme of treatment for the present will be as follows:

(1) Protein hypersensitiveness or *true anaphylaxis* in which antigen and antibody reactions are involved, in which the inciting substance is unquestionably an antigen and in which, as far as we know, inheritance from both parents plays no rôle.

In close relation to this we will discuss (a) *serum sickness*, in the interpretation of which analogy with anaphylaxis has been denied, particularly by Coca, for reasons that will be set forth, and the so-called (b) *food idiosyncracies* in which the inciting substance is clearly an antigen but in which the important factor of true inheritance seems to be involved, and (c) Hay Fever in which the antigenic nature of the inciting substance is reasonably an open question.

(2) *Idiosyncrasies to non-antigenic substances*, drugs, etc., and allied phenomena, in which no antigenic properties can be demonstrated for the inciting substances, as such, in which the factor of inheritance seems to be a very important influence, and in which the possibility of passive transfer is at least doubtful.

(3) Bacterial hypersensitiveness and specific reactions, such as the tuberculin, typhoidin, etc., with a consideration of Bacterial Anaphylatoxin.

(4) Toxin hypersusceptibility.

(5) The primary toxic action of normal serum.

(6) Discussion of coördinating facts.

HYPERSUSCEPTIBILITY TO PROTEINS, OR TRUE ANAPHYLAXIS

Although some of the criteria by which True Anaphylaxis must be characterized will become clear only after a further study of succeeding sections, we believe it well to state them in this place, more or less as they are well stated in our opinion by Wells in the article referred to. The criteria which should, according to Wells, be met in order that a condition may be spoken of as True Anaphylaxis are

(1) The observed toxicity of the injected material must depend upon the sensitization of the animal, that is, the substance should not produce similar symptoms in the non-sensitized animal on first injection.

(2) The symptoms produced must be those characteristic of anaphylactic intoxication as observed in the usual reactions with typical soluble proteins, being, therefore, the same for all antigens with the same species of animal, but differing characteristically in various species of animal.

(3) It should be possible to sensitize a normal animal passively by serum transfer from a sensitized (or immunized) animal.

(4) When guinea pigs are used, the typical uterine reaction should be demonstrable by the Dale method.

(5) Desensitization under proper conditions should be possible.

We omit some of the other criteria stated by Wells because we think the above are sufficient to characterize the condition and comprise the fundamentally important ones.

In discussing anaphylaxis at first in this limited sense, we wish to make entirely clear, however, that we do not mean to imply that our own opinion necessarily agrees with the assumption that there is absolutely no connection between protein hypersusceptibility and other forms, as maintained by Coca. As we proceed in the discussion of the various phenomena, it will become clear that, in spite of many apparent differences in mechanism, there may still be certain fundamental physiological similarities between all forms of changed reaction on the part of the animal body to extraneous substances.

The Antigen.—Since, as we shall see, protein anaphylaxis is dependent upon a reaction between an antigen and its antibody, it is but natural that the sensitizing substances should be in every way similar to other antigens. We may state briefly, therefore, that any substance which can induce antibody formation by injection into an animal, is capable of being used as a sensitizing or anaphylactic antigen. Such a statement, as we have seen in other places, is synonymous with saying that all proteins may be regarded as possible

anaphylactic antigens. It must not be forgotten, however, that there are certain chemically true proteins that have no antigenic properties. Wells has gone into this matter thoroughly in the review of the subject to which we have referred above. The best known of the true proteins which have no antigenic properties is gelatin. According to Starin,[14] whose work Wells regards as sound, the chief difference between gelatin and the antigenic proteins depends upon the deficiency in gelatin of tryptophane and tyrosin, and a low content of phenylalanine; and Wells believes it possible that the deficiency in aromatic amino acids may be the important factor. Wells and Osborne[15] have studied these questions particularly with zein from corn, in which glycin and tryptophane are lacking, and with gliadin and hordein in which glycin, lysine, arginine and histidine are deficient. Wells reasons that the fact that these proteins, so poor in diamino acids, are excellent antigens, while certain protamines which are composed to a large extent of diamino acids have no antigenic action, may signify that the three diamino acids mentioned are of very little antigenic importance. He mentions this merely as a suggestion.

As far as the question of the antigenic activity of protein cleavage-products is concerned, the problem in regard to anaphylaxis is enentirely analogous to that bearing on the antigenic nature of such substances in general. The anaphylactic reaction has been the one, however, very largely used of recent years for the determination of antigenic properties with doubtful antigens, largely because of its extreme delicacy and, for this reason we discuss the subject in this place. Fink,[16] working in Wells' laboratory, studied particularly the cleavage products from hydrolyzed egg-white and found slight antigenic activity for the fractions precipitated at three-quarters and complete saturation with ammonium sulphate, but not for those obtained with lower concentrations of the salt. The question we are discussing here must not be confused with the problems of peptone shock, etc., which will be taken up later, since, at present, we are interested not so much in the ability of various protein cleavage-products to induce toxic, anaphylaxis-like effects upon injection into animals, but rather in the properties of such substances to sensitize animals to subsequently repeated injection. Among the most important contributions to this particular subject is the work of Zunz[17] who obtained heteroalbumose and protoalbumose by peptic and tryptic digestion of fibrin, and found that both of these substances sensitized and intoxicated guinea pigs and rabbits. He was forced to use considerable dosea and his shock reactions were limited in degree. Also,

[14] Starin. *Jour. Inf. Dis.*, 23, 1918, p. 139.
[15] Wells and Osborne. *Jour. Inf. Dis.*, 8, 1911, p. 66.
[16] Fink. *Jour. Inf. Dis.*, 25, 1919, p. 97.
[17] Zunz. *Zeit. f. Immunitäts.*, 16, 1913, p. 580.

specificity was not as marked as with proteins. Attempts to repeat Zunz's work, by Friedberger and Joachimoglu [18] were unsuccessful, and they regard Zunz's results as due to the primary toxic action of the large doses of beef serum used by him in testing beef-albumose sensitized animals. Hailer [19] carried out an extensive investigation on a similar subject with digestion products of beef and hog muscle. He obtained a certain amount of actual sensitization, but found that specificity was lacking and that the sensitization was never intense. Other investigations, such as those of Schmidt [20] were unsuccessful, and Wells [21] found that even digesting bovine serum as long as 16 months with trypsin did not result in a complete loss of coagulable material, in spite of the fact that it gave no Biuret reaction. The subject is an interesting and important one, and for a thorough discussion of much of the literature we refer the reader to Fink's article cited above. We believe that in all probability there may be a certain amount of antigenic power in the higher cleavage-products of protein, but that, with the breaking up of the protein molecule, antigenic functions very rapidly diminish, as will be seen in our discussion of the tuberculin reaction, we agree with Landsteiner [22] that molecular size may be quite as definitely involved as chemical structure. This problem will be spoken of again when we describe our own work with bacterial antigens.

There is a considerable literature on the intoxicating effect of protein-split products upon animals sensitized with the whole protein. Thus, Rosenau and Anderson [23] observed toxic effects when they reinjected guinea pigs that had been sensitized with horse serum, with partially digested horse serum. Gay and Robertson [24] sensitized guinea pigs with casein solution, and obtained shock 23 days later by the injection of paranuclein, a partial peptic digestion product of casein. A number of other such experimental findings could be mentioned, but while they are extremely interesting and perhaps of great significance, have little bearing on the subject in hand.

In regard to the antigenic properties of *altered* proteins, again, analogy with antibody reactions in general prevails in anaphylaxis. Work by Obermeyer and Pick, Schittenhelm and Stroebel and more recent work by Landsteiner has shown that proteins altered by iodizing or by the introduction of nitro- and other atom groups, may possess an antigenic action which is altered in its specificity, in that the chemical group introduced now determines the specific reactions. This work will be more particularly discussed in connection with

[18] Friedberger and Joachimoglu. *Zeit. f. Immunitäts.*, 24, 1914, p. 522.
[19] Hailer. *Arb. a. d. Kais. Ges.*, 1914, p. 527.
[20] Schmidt. *Univ. of Calif. Pub.*, 2, 1916, p. 157.
[21] Wells. *Jour. Inf. Dis.*, 6, 1909, p. 506.
[22] Landsteiner. *Biochem. Zeit.*, 43, 1919, p. 106.
[23] Rosenau and Anderson. *Hyg. Lab. Bull.*, 36, U. S. P. H. Service, 1907.
[24] Gay and Robertson. *Jour. Exper. Med.*, 16, 1912, p. 470.

drug idiosyncrasies, since Wolf-Eisner and others have suggested that so-called drug idiosyncrasies may be due to alterations of the body protein by the drug in such a way that the new complex antigen may act like an altered protein after the drug has been injected.

Another point which is of great importance in connection with antigens in general, and particularly as it affects anaphylaxis, is the factor of racemization. Ten Broeck,[25] working with proteins racemized by Dakin's method, that is, so changed in structure that they are no longer susceptible to enzyme action and are optically altered, found that they no longer were antigenic in anaphylactic reactions. This point is of considerable importance in working with protein extracts in which alkalin extraction is necessary.

Here, as in other phases of the subject the question of the possible antigenic properties of lipoids has been raised, but again without definitely positive results. Pick and Yamanouchi [26] extracted beef and horse sera with alcohol, and evaporated and redissolved the solutions until neither contained coagulable protein nor gave the Biuret reaction. With this material they obtained a few positive anaphylactic experiments. Similarly curious are the results of Bogomolez,[27] who succeeded in sensitizing and producing shock with the lipoids extracted from egg yolks. Although such experiments would tend to persuade us that lipoidal substances may actually have sensitizing, and therefore antigenic, functions, this does not follow necessarily. As Pick and Yamanouchi themselves point out, it is practically impossible to demonstrate with certainty the presence of slight traces of proteins as impurities in lipoid preparations, and we know especially from Rosenau and Anderson's work how minute are the quantities of antigen which still serve to sensitize. It is possible, moreover (a thought developed particularly by Pick and Schwartz [28] and by Landsteiner [29]), that we are dealing in many cases with combinations of protein and lipoid—a form of chemical substance of which very little is known analytically, but the existence of which many biological facts lead us to assume.

Specificity.—That the *anaphylactic reaction is specific* we have mentioned in the brief summary we have given of Rosenau and Anderson's work. These authors use the adjective "quantitative," by which they simply mean to convey that the specificity here is not absolute, any more than it is absolute in the case of any of the known serum reactions. An animal sensitized with a certain variety of protein, animal serum, etc., reacts with disproportionately greater delicacy to a second injection of the same variety than of any other

[25] Ten Broeck. *Jour. Biol. Chem.*, 17, 1914, p. 369.
[26] Pick and Yamanouchi. *Zeitschr. f. Immunitätsforschung*, Vol. 1, 1909.
[27] Bogomolez. *Zeitschr. f. Immunitätsforschung*, Vols. 5 and 6, 1910.
[28] Pick and Schwartz. *Biochem. Zeitschr.*, 15, 1909.
[29] Landsteiner. Referat. "Weichhardt's Jahresbericht," 6, 1910.

substance. In fact, apart from a few cases mentioned by Gay and Southard, there are not many instances of marked non-specific anaphylactic reactions. Still we would expect here, as in other serum reactions, a certain limitation in the degree of specificity, and Otto recommends the less delicate subcutaneous method of testing for all experiments in which questions of specificity are involved. This point we will touch upon a little later.

An interesting addition to our knowledge of such specificity was made by experiments of Rosenau and Anderson, which showed that a guinea pig could be rendered separately sensitive at one and the same time to blood serum, eggwhite, and milk, reacting specifically to each on second injection.

In considering the specificity of antigens, it must be remembered that the ordinary substances used for anaphylactic and other antibody reactions, such as eggwhite, animal serum, etc., are really mixtures in which a number of different proteins may be represented and the various fractions of such a complex substance may possess individual specificity. An extremely interesting instance of this is found in the work of Dale and Hartley[30] who, using the guinea pig uterus technique of Dale, which is extremely sensitive, and using as antigens euglobulin, pseudo-globulin and albumin of horse serum, found that these horse serum fractions could be made to sensitize separately. Similarly, separate and individually specific antigens have been obtained from other proteins, such as egg albumen and plant proteins, particularly by Wells and Osborne. The last named authors, too, have obtained some extremely interesting results in their attempts to correlate chemical structure with specificity. Their opinion is based chiefly upon their experiments with vegetable proteins in which interaction between chemically related proteins could be demonstrated by anaphylactic reactions. Their experiments are of unusual importance, but the entire problem is still in its beginnings. In this connection, the more recent investigations of Landsteiner on proteins altered by the introduction of diazo bodies, and other atom groups, are extremely important in that they show, as Doerr also points out, that relatively slight changes in the molecule may considerably change and even completely mask specific reactions to the native protein. In appraising this work, Doerr in his most recent article, sums it up very well by stating that it seems quite certain at the present time that one and the same protein antigen may be altered into a considerable number of substances of individual specificity, and that by similarly altering a number of different proteins similarly, that is, in chemical treatment, a closely related specificity may be produced in them. Along lines, such as these, we may eventually hope to obtain a certain amount of insight into the hitherto mysterious problem of specificity.

[30] Dale and Hartley. *Biochem. Jour.*, 10, 1916, p. 110.

In anaphylaxis, again analogous to antibody reactions in general, the specificity, as a rule, is one of species. In other words, the protein of any animal is specific for the proteins of its particular species generally, there being definitely similar characteristics in the body proteins of animals of like species, which, though chemically indefinable, are nevertheless delicately determinable by biological reactions. In considering specificity of precipitins, however, we have seen that there are exceptions to the specificity of species expressed in the phenomenon of so-called organ specificity. The same thing has been shown for anaphylaxis. Kraus, Doerr and Sohma [31] were able to show that animals sensitized with protein from the crystalline lens were hypersusceptible to lens protein generally, whether this came from the species from which the original lens was taken, or whether some other variety of animal had furnished it. On the other hand, animals so sensitized, while hypersusceptible to lens protein, did not react to injections of homologous blood.[32] In other words, this organ contains a characteristic variety of antigen (protein) peculiar to this kind of organ throughout the different animal species, but not common to other tissues and organs of the same animal. Results similar to these were obtained by von Dungern and Hirschfeld [33] in the case of testicular protein, although here the phenomenon seemed to be less rigidly organ-specific than in the preceding case. These writers worked not with the systemic anaphylactic reaction, but with the localized (allergic) reaction described above as the phenomenon of Arthus. They injected extracts of the testicular materials into the ears of rabbits and incidentally made the very curious observation that pregnant females would not infrequently react to a first injection without previous sensitization.

Of great importance also in connection with the subject of *organ specificity* is the further claim of Uhlenhuth and Haendel [34] that animals can be sensitized with their own lens protein, an observation which opens the possibility of other forms of "autosensitization" and consequently has given much opportunity for clinical speculation. Rosenau and Anderson,[35] indeed, have found that guinea pigs can be sensitized by means of extracts of guinea pig placenta. They have applied this to the possible explanation of eclampsia, and similar reasoning, as we shall see, has been utilized in many other conditions. Attempts have also been made to show, by the anaphylactic reaction, that the tissue of malignant tumors possess such "tissue-specific" or "organ-specific" qualities. Yamanouchi,[36] indeed, claims

[31] Kraus, Doerr and Sohma. *Wien. klin. Woch.*, No. 30, 1908.
[32] Andrejew. *Arb. a. d. kais. Gesundh. Amt.*, Vol. 30, 1909.
[33] Von Dungern and Hirschfeld. *Zeitschr. f. Immunitäts.*, 4, 1910.
[34] Uhlenhuth and Haendel. *Zeitschr. f. Immunitäts*, 4, 1910.
[35] Rosenau and Anderson. *U. S. Pub. Health and M. H. S. Hyg. Lab. Bull.*, 45.
[36] Yamanouchi. *C. R. de la Soc. Biol.*, Vol. 66, 1909, p. 754.

to have shown this, but his results were not confirmed by Apolant,[37] and the writer has carried out a series of entirely negative experiments upon the same subject. However, in view of the great difficulty of obtaining any kind of anaphylactic reaction in mice, the animals in which the tumor experiments were carried out, there is little information to be obtained from negative results of this kind.

The delicate quantitative method of studying problems of specificity, which the reaction of anaphylaxis supplies, has further served to revive the unsettled question of the "organ-specific" properties of the tissues of such organs as the liver, spleen, kidney, blood, etc. Indeed, Pfeiffer,[38] has published results which would seem to encourage the belief of the existence of such specificity. However, Ranzi [39] had previously obtained entirely negative results, and Pearce, Karsner, and Eisenbrey [40] have recently made a careful inquiry into the same problem, failing to confirm Pfeiffer's claims.

By a similar process of reasoning Elschnig [41] has attempted to explain sympathetic ophthalmia. He claims to have shown that the laws of organ specificity apply to the proteins (especially the pigment) of the uveal tract. The destruction and absorption of injured uveal tissue, according to him, induce the formation of organ-specific antibodies by which the remaining uveal structures of the same, as well as of the opposite, eye are sensitized. The consequence is a "sympathetic" inflammation which "is to be regarded purely as an anaphylactic reaction."

These investigations, while regarded with much scepticism by immunologists in general, seem to be yielding confirmatory results in the hands of Allan Woods who has begun to carry out the principles of Elschnig's reasoning on human beings suffering from sympathetic ophthalmia. Although nothing definite can be said about this work, as yet, it seems increasingly worth putting to practical test in view of Woods' beginning.

In this, then, as well as in other respects, the *substances by which animals may be sensitized are entirely similar to antigens in general.*

Active Sensitization.—Active sensitization is a term analogous to active immunization in that it signifies that the animal is sensitized by the injection of the antigen, and develops its sensitiveness in physiological response to the injection. This is the original manner in which sensitization was first accomplished, and is in contrast to *passive sensitization* which we will discuss directly, in which the animal is rendered sensitive not by its own antibody formation, but by virtue of antibodies transferred to it from the actively sensitized

[37] Apolant. *Zeitschr. f. Immunitätsforschung,* Vol. 3, 1909.
[38] Pfeiffer. *Zeitschr. f. Immunitätsforschung,* Vol. 8, 1910.
[39] Ranzi. *Zeitschr. f. Immunitätsforschung,* Vol. 2, 1909.
[40] Pearce, Karsner, and Eisenbrey. *Jour. Exp. Med.,* Vol. 14, 1911.
[41] Elschnig. *Von Graefe's Archiv. f. Ophthal.,* Vol. 75, p. 459; Vol. 76, p. 509; Vol. 78, p. 549.

one. Thus, the terms are in complete analogy to the similar ones used in connection with immunization.

Active sensitization, then, consists in the injection of the antigenic substance into the animal, as a result of which it gradually becomes specifically hypersensitive to the injected substance. As far as the *manner of injection* is concerned, we can state, briefly, that the conditions are again exactly analogous to those prevailing in immunization in that any manner of administration in which the antigen may come in contact with the tissue cells of the body without previous alteration by digestive ferments (in other words, in its antigenic condition) may lead to sensitization. This is synonymous with saying that any manner of introduction by which the antigen may lead to antibody formation, may sensitize. Thus, sensitization may be accomplished by subcutaneous, intravenous, intracardial, intramuscular, or any other *parenteral* method of injection. It is probably possible, also, under unusual circumstances, that antigen may pass through mucous membranes like those of the eye and nasopharynx, and thereby sensitize. Whether or not sensitization through the intestinal canal is possible, is a question that has been much discussed, especially in an attempt to explain hypersensitiveness to various food substances in young infants and children, in whom it is assumed that the absorption of unchanged foreign protein may possibly take place through the intestinal mucosa, either during the very early days of life, or later, by reason of injury of the mucosa by the intestinal disease accompanying various forms of malnutrition and diarrhea. Recent work by Schloss and others seems to indicate that protein may pass into the body in its antigenic form in this way. Such a possibility becomes more plausible when we consider the very minute amounts of antigen which may lead to sensitization.

Rosenau and Anderson, in their earliest paper, report success in sensitizing guinea pigs by the feeding of horse meat and horse serum. McClintock and King [42] failed to confirm this, and the observations of other writers seem to bear them out. However, when we consider that Ascoli, Oppenheimer, and others have shown that proteins fed to animals in large quantities may be subsequently demonstrated not only in the circulating blood but occasionally even in the urine by means of the precipitin reaction, there seems to be little room for doubting that antigen may enter the circulation unchanged, though possibly only under abnormal local conditions of the intestine. This, together with Rosenau and Anderson's demonstration of the extremely small amount of antigen necessary to sensitize, furnishes all the conditions necessary for anaphylaxis by way of the intestinal canal.

[42] McClintock and King. *Jour. Inf. Dis.*, 3, 1906. See section on normal antibodies.

A study made by Lesné and Dreyfus [43] seems to us to have explained the contradictory results of other workers on this phase of the problem. Without being able to associate the destruction of the sensitizing function with either the gastric or pancreatic secretions, they were nevertheless successful in showing that sensitization could be carried out regularly if the antigen were injected after laparotomy into the large intestine, whereas similar injections into the stomach or small intestine were negative. In these experiments we must take into consideration that the conditions following laparotomy, such as temporary intestinal atony and congestion, may have exerted considerable influence upon the positive outcome of their large intestine injections. Whereas they do not, therefore, permit us to assume the possibility of sensitization through the normal alimentary canal, they nevertheless confirm the assumption of the possibility of sensitization by this path under the influence of the abnormal local conditions incident to injury or disease.

In this connection Besredka's [44] experiments on the production of anti-anaphylaxis by the intestinal administration of protein are of interest. He found that, if sensitized animals were given 5 c. c. of the antigen (milk) by rectum, they were thereby protected from the reaction following in controls upon a second injection. In his later experiments with egg white it appeared that the protection could also be conferred by mouth, but that in this case it developed more slowly, it being necessary to wait two days after injestion before the anti-anaphylaxis had developed sufficiently to protect. Since attempts by mouth were not as rapidly successful as those *per rectum,* it is clear that these facts are in keeping with Lesné and Dreyfus' results in showing that the antigen is probably absorbed chiefly or solely from the large intestine. In Lesné and Dreyfus' experiments the sensitizing dose was given into the intestine, the toxogenic or second dose being administered intravenously, and since, as we shall see, minute doses may suffice to sensitize, whereas 100 or more times the sensitizing amount is necessary to produce intoxication, it is easy to understand why sensitization followed in Lesné and Dreyfus' work, but no toxic effects followed in the experiments of Besredka. Furthermore, the slow absorption from the intestine in these experiments explains the development of anti-anaphylaxis in Besredka's work, in that they are, in this respect, analogous to later experiments of Friedberger, cited below, in which it was shown that sensitized guinea pigs, which could (in controls) be killed by rapid intravenous injection of 0.1 c. c. of antigen and less, would withstand without symptoms many times this amount when it was gradually administered by slow injection covering an hour or longer.

The *quantities of antigen* which are necessary to sensitize may

[43] Lesné and Dreyfus. *C. R. de la Soc. Biol.,* Vol. 70, p. 136, 1911.
[44] Besredka. *C. R. de la Soc. Biol.,* Vol. 65, 1908; Vol. 70, 1911.

vary tremendously. The underlying fact governing this can probably be stated best by saying that all that is necessary is to introduce an amount of antigen sufficient to initiate antibody production. This will, of course, vary within very wide limits for the several antigenic substances used, depending upon the concentration of the antigenic soluble protein in the preparation. When working with substances as antigenically potent as horse serum, Rosenau and Anderson [45] obtained definite sensitization after the injection of as little as one-millionth of a cubic centimeter of horse serum. Their experiments with horse serum generally range from a sensitizating dose of this minute quantity to as high as 10 c. c. in guinea pig experiments. Quantities almost as low as those reported by Rosenau and Anderson for guinea pig sensitization have been successfully used by Doerr and Russ,[46] and still lower quantities have sensitized in the hands of Wells when using crystallized egg albumen.

Such minute quantities are not, however, the most favorable for active anaphylactic sensitization, and are mentioned only to show how extremely delicate the reaction may be. It is also worth mentioning, in passing, although we will refer to it again, that the second injection which elicits shock, must always be considerably larger than these small sensitizing amounts. To obtain constant and reliable results in guinea pigs, a single sensitizing injection, ranging in amounts from 0.01 to 0.25 c. c., is usually sufficient. These amounts as given, should be taken to apply only to guinea pigs. We will discuss variations as observed in other animals directly.

Incubation Time.—The hypersensitive state may supervene at varying periods after the sensitizing injection, modified largely by the amount of antigen injected. Though often so stated, it is not likely that the route of injection matters very much, so long as the administration is parenteral. For, even though intravenous or intracardial injection means that the antigen comes into almost immediate contact with the tissues, subcutaneously injected antigen probably does not lag much more than 72 hours behind at most, and some of the antigen will probably get into the circulation in such cases almost immediately. The quantity, however, seems to influence the length of the incubation time considerably. At first there was an idea based upon the earlier observations of Rosenau and Anderson and of Otto [47] that the length of incubation time was inversely proportionate to the size of the sensitizing dose; in other words, moderately small quantities would sensitize more rapidly than very large amounts. Later experiments of Rosenau and Anderson, however, seem to show that

[45] Rosenau and Anderson. *U. S. P. H. Bulletin*, No. 29, 1906, and No. 45, 1908.

[46] Doerr and Russ. *Zeitschr. f. Immunitäts.*, 2, 1909.

[47] Otto. Cited from Kolle & Wassermann *Handbuch*, Ergenzungsb., No. 2, 41.

this relation is not as definite as at first assumed. In the tables given by them guinea pigs receiving 0.01 c. c. reacted severely after 14, 17, and 155 days; others, receiving 1 c. c., after 14, 17 and 155 days; and, again, another series sensitized with 8 c. c. reacted severely after similar intervals. All of these series reacted but mildly after 245 days, showing apparently that the anaphylaxis, contrary to general belief, does not last so much longer after the larger than after the smaller sensitizing doses. In general, we can say that, while very large doses of antigen may perhaps increase the length of the incubation time, perhaps because of the prolonged presence of antigen in the circulation and the partial neutralization of the first formed antibodies by this residue, no such prolongation results from moderate doses. Below a certain limit, it is probable that the smaller the dose, the longer the incubation time, because of the slower development of antibody formation in consequence. Repeated injection of guinea pigs with the same antigen during the period of incubation, as one would expect, prolongs the incubation time because of the partial desensitization which follows each new injection. This will become clear on further discussion below. Thus, in general, we may say that guinea pigs actively sensitized with 0.01 c. c. and more of a serum antigen, may begin to grow definitely hypersensitive within 5 to 7 days, not reaching their most highly sensitive condition, however, until the 8th to the 14th day, after the injection.

As stated above, what has been said so far refers to guinea pigs. Strange as it seems, other animals cannot be sensitized in exactly the same way.

Rabbits are best sensitized by repeated injections administered at intervals as in immunization. A single injection may occasionally produce the anaphylactic state in rabbits if a sufficient amount of antigen is injected in the first place, and the test injection made in between two and three weeks. However, such an experiment is not often successful. The best procedure in rabbits is to inject moderate quantities of antigen intraperitoneally or intravenously at intervals of 5, 6, or 7 days, as in the production of a high titer precipitating serum. It is not then very easy to know exactly when the best time for reinjection has come. Usually, when in their most sensitive condition, rabbits have at the same time a considerable amount of precipitating antibody in the serum and this is one of the reasons, as we shall see, for the earlier ideas that in rabbits the reaction took place entirely in the circulation. As a matter of fact, as many observers have shown, the rabbit is not merely so sensitive to the anaphylactic experiment as the guinea pig.

In dogs, acute anaphylaxis cannot be produced by a single injection, though protracted symptoms eventually ending in death, may be obtained after a single sensitizing injection. Richard Weil [48] did

[48] Weil, Richard. *Jour. Immunol.,* 2, 1917, p. 525.

finally succeed in producing acute shock in dogs by giving a first subcutaneous injection of 5 c. c. of the antigen, and following this after 3 days with a similar amount intravenously administered. Something over 2 weeks later, 20 c. c. of the serum brought on acute shock with death in one-half hour.

Repeated studies on monkeys seem to show that the lower monkeys are certainly not easily sensitized under any conditions. Our own studies on this subject with Macaccus Rhesus and Ringtail monkeys seem to show that a single injection of horse serum may produce no anaphylactic reactions whatever, even though the tests were controlled by the delicate Dale uterine method. Mild anaphylactic symptoms may be elicited after repeated and prolonged treatment with the antigen.[49]

Reinjection for the Production of Anaphylactic Shock.—The antigen may be administered for the purpose of eliciting anaphylactic shock in an animal in a number of ways. The best method of obtaining severe symptoms is the intravenous in which the maximum concentration of the injected antigen comes into contact with the sensitized tissues suddenly. In animals that are not particularly sensitive to anaphylactic reactions, rabbits, dogs, etc., the intravenous method is the only one with which acute death can be obtained with any degree of regularity. Intracardial injections, as first practiced by Rosenau and Anderson, Lewis [50] and others in anaphylactic experiments, amount to about the same thing as intravenous injection, and has the disadvantage of a more difficult technique and some uncertainty in regard to getting the total quantity injected into the cavities of the heart. When the injections are made subcutaneously, considerably larger amounts must be given in order to obtain the same results. The subcutaneous injections into sensitized rabbits produce marked local reactions consisting of oedema and sometimes eventual necrosis, the state of affairs spoken of above as the Arthur phenomenon. This does not occur in the same way in guinea pigs and other animals, though the explanation for the peculiarity of the rabbit in this respect is not clear. It is suggested by Coca that perhaps the local reaction on subcutaneous injection in rabbits indicates a general capillary reaction in these animals similar to the vascular contraction demonstrated by him in the pulmonary system. Local reactions on subcutaneous injection may appear in guinea pigs, man, and rabbits, in the form of varieties of skin reactions which will be discussed in a subsequent section. The generalized reaction, however, is identical with that following intravenous injection, only less severe, unless large doses are given. Shock may be obtained in guinea pigs also by intraperitoneal injection. Subdural and intracerebral injections were practiced extensively for a time in connection with

[49] Zinsser. *Proc. Soc. Exper. Biol. & Med.*, 18, 1920, p. 57.
[50] Lewis. *Jour. Exper. Med.*, 10, 1908.

Besredka's [51] earlier theory of anaphylaxis. Besredka and Steinhardt [52] who began their studies soon after the first publication of Rosenau and Anderson,[53] came to the conclusion that the most rapid and conclusive method of producing anaphylactic shock in animals consisted in such brain injections. Whether or not we have reliable evidence of anaphylactic symptoms supervening upon contact of the antigen with the mucous membrane of sensitized animals, is an important question which will be discussed in a subsequent section in connection with asthma and hay fever.

It is important to note that the quantities of antigen necessary for the production of anaphylactic symptoms or shock can never be reduced to the minute amounts with which sensitization has been possible. This is of considerable importance in judging some of the earlier theories, such as that of Besredka, and that of Gay and Southard, whose ideas involved the supposition that the substance in the antigen which sensitized was not identical with that which elicited shock on subsequent injection. The point will be taken up later, but it is worth noting in this place that while it is possible to sensitize with doses as small as one-thousandth of a cubic centimeter and less, considerably larger quantities are needed for the development of shock.

Duration of Hypersensitive State.—The experiments of Rosenau and Anderson cited above have shown in a number of cases that mild but definite anaphylactic reactions may be elicited in guinea pigs sensitized with horse serum as long as 245 days after the injection, there being no particular difference in this respect between animals sensitized with moderate doses or those sensitized with large doses. A few guinea pigs sensitized with toxin-antitoxin mixtures by them, gave positive reactions after as long as 732 days, and in one case, more recently reported, they obtained a reaction after 1096 days. It is not at all unlikely that individuals once sensitized may remain so for life, the sensitiveness gradually fading unless new contact with the antigen occurs. Temporary desensitization, as we shall see, merely interrupts this condition for a limited time, the individual returning to eventual hypersusceptibility.

Passive Sensitization

Up to the present time we have confined ourselves to the description of the basic anaphylactic experiment, which is spoken of as *"active sensitization"* in analogy to the expression "active immunization," since, like the latter, it conveys the conception that the state

[51] Besredka. *Ann. de l'Inst. Past.*, 1907, pp. 777, 950; 1908, p. 496; 1909, pp. 166, 801; *Bull. de l'Inst. Past.*, 1908, No. 19, No. 20, No. 21; 1909, No. 17; *C. R. Soc. de Biol.*, 65, 1908, p. 478, and 67, 1909, p. 266.

[52] Besredka and Steinhardt. *Ann. de l'Inst. Past.*, 1907, pp. 177, 384.

[53] Rosenau and Anderson. *U. S. P. H. Bulletin*, No. 50, 1909.

of hypersusceptibility (like the immunity in active immunization) is here acquired by reason of physiological changes directly induced in the treated animal in reaction to the first injection of the foreign antigen. There is another method of inducing hypersusceptibility which, in continuance of the analogy to immunization, is spoken of as *"passive anaphylaxis,"* since it consists in transferring the hypersusceptible condition to a perfectly normal animal by injecting into it serum from an actively sensitized one. The normal animal is thus merely the passive recipient of the reaction bodies produced in the sensitive animal by preliminary treatment.

That such a passive transference of anaphylaxis is possible was shown by a number of investigators almost simultaneously and M. Nicolle,[54] in February, 1907, published a study on the phenomenon of Arthus in which he showed that, if the serum of a hypersusceptible rabbit (sensitized with horse serum) was injected into a normal rabbit, the recipient was rendered sensitive, so that the subcutaneous injection of horse serum, made 24 hours later, produced typical infiltrations. Richet[55] soon after this succeeded in transferring hypersusceptibility toward mytilocongestin (a mussel poison) from a sensitized to a normal dog by injecting considerable amounts of the blood from the former into the latter. In this case, too, the hypersusceptibility of the second dog did not appear until one or two days after the injection of the blood. At almost the same time Otto[56] and Friedemann[57] independently succeeded in transferring serum anaphylaxis from hypersusceptible to normal guinea pigs in a similar way. Experiments of Gay and Southard,[58] published during the same year, may possibly be also interpreted as instances of passive anaphylaxis, although their experimental procedure renders this doubtful, even in their own opinions. They injected 0.1 c. c. of serum from both sensitive and refractory guinea pigs into normal animals and followed this, after 10 days, with injections of antigen. The fact that such animals reacted may be interpreted in a number of ways. They themselves regarded the hypersusceptibility which the injected animals developed as a "purely active one," and it is more than likely that this was the case, the recipient animals being actively sensitized by traces of antigen remaining unassimilated in the blood of the actively sensitized donors. In the following year (1908) the facts of passive sensitization were rapidly confirmed and extended by Besredka,[59] Lewis,[60] and others,[61] and information of

[54] M. Nicolle. *Ann. de l'Inst. Past.*, Vol. 21, 1907.
[55] Richet. *Ann. de l'Inst. Past.*, Vol. 21, 1907.
[56] Otto. *Münch. med. Woch.*, No. 34, 1907.
[57] Friedemann. *Münch. med. Woch.*, No. 49, 1907.
[58] Gay and Southard. *Jour. Med. Res.*, Vol. 16, 1907.
[59] Besredka. *Ann. de l'Inst. Past.*, Vol. 22, 1908.
[60] Lewis. *Jour. Exp. Med.*, Vol. 10, 1908.
[61] Kraus and Doerr. *Wien. klin. Woch.*, No. 28, 1908.

the greatest value for the comprehension of the anaphylactic reaction was obtained.

Otto showed that passive sensitization could be carried out with the serum of an actively sensitized animal 8 days after the antigen injection, at a period when this animal itself had not yet become hypersusceptible. He also showed that the passive transfer of anaphylaxis need not be confined to animals of the same species, but that guinea pigs could be rendered passively anaphylactic with the blood serum of sensitized rabbits. From the work of Gay and Southard,[62] moreover, it appeared that not only by the blood of sensitive animals can anaphylaxis be transferred, but that this can also be done by injecting the blood of animals that have once been sensitive but have subsequently been rendered antianaphylactic or refractory. Analogous to this observation is the fact observed by these authors as well as by Friedemann that the young of antianaphylactic mothers are not refractory but hypersusceptible. This observation is unquestionably correct, and has been confirmed by several other works. It has had no inconsiderable bearing upon our theoretical understanding of anaphylaxis.

It was soon found out, too, that hypersusceptibility was conveyed not only by the sera of sensitive and of refractory animals, but that it could likewise be transferred by the precipitating sera of animals systematically immunized with a foreign proteid.

This method was later employed by Doerr and Russ [63] in their quantitative studies on the relations between anaphylactic antigen and antibody. We are confronted, then, with the facts that animals may be passively sensitized:

(a) by the serum of a sensitized animal.

(b) by the serum of an animal not yet sensitive—in the preanaphylactic period (8th day, Otto).

(c) by the serum of an antianaphylactic animal.

(d) by the precipitating serum of an "immunized" [64] animal.

Lewis further showed that normal guinea pigs could be rendered hypersusceptible with the blood of congenitally sensitive animals.

In order to transfer hypersusceptibility from one animal to another in this way, it is not necessary that both animals be of the same species. As a matter of fact, convenience in laboratory experiments has led to the almost general practice of using the serum of immunized rabbits for the transference of passive anaphylaxis to guinea

[62] Gay and Southard. *Jour. Med. Res.*, Vol. 18, 1908.

[63] Doerr and Russ. *Zeitschr. f. Immunitätsforschung,* Vol. 3, 1909.

[64] We must never forget that the term "immunized" as applied to animals treated with harmless protein is an analogy and not absolutely correct. Such animals, though probably capable of assimilating larger quantities of foreign injected protein than normal ones, and this more rapidly, may nevertheless be not a whit more tolerant of the antigen—sometimes even extremely sensitive and vulnerable.

pigs. A great many different combinations, however, have been employed and the antibody-containing serum of rabbits, dogs, man and horses have been successfully employed for the passive sensitization of guinea pigs. It is likely that any serum which contains antibodies and is not normally toxic for the injected species, may passively sensitize. Doerr notes definite exceptions which he summarizes from the literature, stating that transference was unsuccessful in attempts to use the serum of birds on mammalia, and vice versa, that of mammalia on birds. He also records negative results in similar attempts to sensitize white mice with the serum of rabbits and guinea pigs. In the latter case, the difficulty of producing anaphylactic sensitiveness in mice under any circumstances may account for this. In those instances in which species as far separated as mammals and birds have been used, it is quite possible that no relationship between any of the constituents of the foreign serum and the cells of the host is established. A few years ago we attempted to do some single cell anaphylactic experiments in which we tried to absorb anti-horse serum upon living paramœcia and amoebæ with complete failure. The constitution of the proteins in species far removed from each other seems to render difficult, or perhaps entirely preclude combinations of cellular substances and the heterologous antibodies.

Passive sensitization is carried by the blood serum purely, since, in ordinary cases, as Rosenau and Anderson have shown, the blood corpuscles and tissues of a sensitive animal do not convey the hypersusceptibility. An exception to this will be noted later when we come to discuss Bail's experiments on the passive transfer of tuberculin sensitiveness.

Passive sensitization, once established, may persist for as long as 3 or 4 weeks, though Rosenau and Anderson found that animals tested 26 days after treatment reacted but weakly. In the young of anaphylactic mothers Otto has observed positive reactions as long as 44 days after birth, though fatal results were obtained in pigs only a few days old. In general passive sensitization lasts longer if conferred with homologous rather than heterologous anti-serum.

To summarize the matter briefly, we may state that *passive sensitization may be accomplished with any serum that contains antibodies, and that the power of such a serum to convey passive sensitization is in direct proportion to its antibody concentration;* in short the process of sensitization consists in the introduction of antibodies. This fact, which is, of course, of the greatest theoretical importance, will be further discussed in the succeeding chapter.

Throughout the earlier investigations upon passive sensitization the curious fact recurs in the experiments of successive workers that a definite period must elapse between the injection of the sensitive blood and that of the antigen.

Both Friedemann and Otto found that when the serum of a sensi-

tized animal was injected subcutaneously the best results were obtained by administration of the antigen 24 to 48 hours after this. On intraperitoneal injection of the sensitizing serum Doerr and Russ [65] obtained the best results by permitting an interval of 24 hours to elapse, and the same investigators still further shortened this period to 4 hours by injecting the sensitive serum intravenously. Beyond this, the interval could not be shortened with success. Indeed, some writers, notably Gay and Southard, have claimed that the maximum hypersusceptibility in guinea pigs treated with sensitive serum is reached only after 10 or more days, and Rosenau and Anderson, Lewis, and others have obtained results which seemed to point in the same direction. However, as we have already indicated, the testing of animals so long after the injection of sensitive serum leaves us in doubt whether we are dealing with true "passive" transference of anaphylaxis or with active sensitization due to traces of antigen carried over with the serum of the sensitive animal.[66]

From these observations the natural deduction was made that the anaphylactic symptoms were the result of cellular occurrences, and that the antigen could act only after the sensitizing substance (however conceived) had become attached to certain cells, probably to those of the central nervous system. It was thought that a meeting of antigen and the sensitized body in the circulation would result in no reaction; that, in other words, the effective reaction was not a direct, but an indirect, one after the anaphylactic "antibody" of the sensitive serum had become bound to the cells. It will be necessary to recur to this problem when we discuss the various theories of anaphylaxis, where we will see that this point has been one of the crucial ones in the controversy between the two main directions of thought on anaphylaxis.

To *summarize* what we have so far said, then, about passive anaphylaxis, the facts elucidated by all these investigations were, in the first place, that *the state of hypersusceptibility can be specifically transferred to a normal animal with serum which contained antibodies,* whether it was derived from an animal in the condition of hypersensitiveness, or from one in which no actual hypersensitiveness could be elicited, but which had been treated actively with the serum protein in question. Such transfer could take place not only within

[65] Doerr and Russ. *Zeitschr. f. Immunitätsforschung,* Vol. 3, p. 181, 1909.

[66] An exception to this, contradicting the then prevailing opinion, were the researches of Weill-Hallé and Lamaire (*C. R. de la Soc. de Biol.,* Vol. 65, July, 1908, p. 141), who showed that, under certain conditions, guinea pigs would react with typical, often fatal, anaphylaxis if injected simultaneously with the serum of sensitized rabbits and the antigen horse serum. According to them, the success of such experiments depended entirely upon the condition of the sensitive serum—that is, the time at which the rabbits treated with horse serum were bled. These researches, with others of a similar bearing, will be discussed in a later section.

the same species, but by the injection of the blood serum of an animal of one species to that of another. The other fundamental fact is that, with very few exceptions, it was noticed that *between the time of the injection of the antiserum and the development of hypersusceptibility, a definite interval must elapse.* The first fact indicated definitely an antigen-antibody union in the body, as the true mechanism of the reaction, the second suggested a cellular seat for the anaphylactic occurrence. This last conception was early suggested by Doerr and Russ, and others, and the interval was supposed to represent the time necessary for the union of what we shall for the present call the anaphylactic antibody, with the cells. This point of view of the necessity for the union of antibody with cells as a necessary criterion of anaphylaxis as we ordinarily see it, has since been definitely confirmed, but soon after the time when this mechanism was first suggested, the issues were somewhat clouded by apparently contradictory experiments of Friedmann and a number of others which led to the formation for a time of two schools of investigators, one adhering to what was spoken of as the "cellular" theory of anaphylaxis, the other maintaining that the entire process took place in the circulation and of which, therefore, we may speak of as the "humoral" school. The researches upon which a final solution of this problem was based and a summary of what we believe the proper point of view should be at the present time, will follow in another section.

We have briefly noted above that the offspring of hypersensitive guinea pigs may be found anaphylactic. Rosenau and Anderson [67] noted this in their early experiments, and the fact has been variously confirmed since then. According to Lewis [68] such inherited hypersusceptibility disappears within the first few months, and may vary in intensity in offspring of the same litter. It appears from everything that we can find in the experimental literature that such inherited hypersusceptibility is based merely upon a passive transfer of antibodies from mother to child. Experimental evidence, also, seems to point to transfer by way of the placenta and not through the colostrum and milk, a fact which bears out recent investigations by Kuttner and Ratner in our own laboratory, who showed that this is entirely the case in connection with the transfer of diphtheria antitoxin from mother to child in human beings, although, as we have stated in discussing their work, it is rash to draw conclusions of this kind from one animal to another, owing to the diverse histological anatomy of the placental structures in different species of animals.

The Antibody Involved.—Doerr and Russ were the first to develop quantitative methods of studying the so-called anaphylactic antibody. They obtained the important result that there was an apparently complete parallelism between the ability of an immune

[67] Rosenau and Anderson. *Loc. cit.*
[68] Lewis. *Jour. Exper. Med.*, 10, 1908.

serum to sensitize passively, and its content in precipitins. Their method consisted in producing precipitating sera in rabbits in the usual way. With these they then passively sensitized guinea pigs, subsequently testing them with antigen 24 hours later. To arrive at quantitative results they developed two reliable methods. These consisted in: 1. Intraperitoneal sensitization of guinea pigs with constant quantities of titrated precipitating serum. Twenty-four hours later intravenous test with diminishing amounts of specific antigen. 2. Intraperitoneal sensitization with diminishing quantities of the titrated precipitating serum, and 24 hours later intravenous tests with constant amounts of antigen.

In this way they showed that there was a direct relationship between the power of a serum to convey anaphylaxis passively and its contents of precipitins. We may elucidate this by an example from their work. They possessed a rabbit serum which gave precipitation with sheep, goat, beef, pig, human, and horse sera, but not with chicken serum. The precipitation titre of this serum for the sera mentioned varied from 1 in 20,000 in the case of sheep and goat sera, to 1 in 100 in the cases of the human and horse sera. When guinea pigs were injected intraperitoneally with 1 c. c. of this serum, and after 24 hours were intravenously injected with the various antigens mentioned above, in decreasing quantities, the sera which were precipitated in the highest dilutions gave anaphylactic shock in the smallest quantities. Those sera for which no precipitin or little had been present gave little or no reaction by this method even where considerable quantities were used. Thus, in animals prepared with 1 c. c. of the antiserum (which precipitated sheep serum in dilutions as high as 1 to 20,000), death was caused by reinjection of sheep serum in amounts as small as 0.006 c. c.; whereas, in similar animals, horse serum which was precipitated in concentrations not higher than 1 to 100 by the passively sensitizing serum, elicited slight symptoms only even when injected in amounts as high as 2 c. c.; and when such animals were reinjected with chicken serum which was not precipitable at all by the antiserum, no reactions whatever could be elicited.

In this, then, we have a definite quantitative analysis which shows a direct relationship between the concentration of antibodies in a serum and its ability to sensitize passively. Doerr and Russ at this time were inclined, therefore, to assume that precipitin and anaphylactic antibody were identical,[69] and this was in keeping with Friedberger's purely theoretical idea that precipitins and the anaphylactic antibody might be identical.[70] It is true that other investigators, notably Hintze [71] found also a parallelism between complement fixing properties and ability to sensitize passively, but in this connection

[69] Doerr and Russ. *Zeitschr. f. Immunitäts.*, 3, 1909, pp. 181 and 706.
[70] Friedberger. *Zeitschr. f. Imm.*, Vol. 2, 1909, p. 208.
[71] Hintze. Quoted from Doerr and Russ, *loc. cit.*

we may refer the reader to a preceding section in this book which deals with the essential identity of antibodies. In that section it may be seen that we ourselves very strongly favor the view that there are two main classes of antigens in nature, one represented by the true toxins and exotoxins, or other active substances, like ricin, aborin, snake venoms, spider poisons, enzymes, etc., which induce a neutralizing antitoxin-like antibody in animals; that inactive antigens, like the proteins, however, give rise to the formation of, in each case, a single sensitizing antibody which either agglutinates, precipitates, fixes complement, opsonizes or sensitizes in the anaphylactic sense, according to the physical and chemical conditions and other environmental factors under which the reaction takes place. We believe, therefore, that the antibody which sensitizes and which, by the way, has a heat stability just like the other protein antibodies, is identical with the general protein sensitizing antibody. If this is not entirely clear, it can easily be understood, at least as far as our opinion is concerned, by referring back to the section alluded to. Most observers have confined themselves to an attempt to either disprove or prove the identity of anaphylactic sensitizing substances and precipitins. Richard Weil [72] found, incidentally, that a specific precipitate obtained by the precipitation of serum with an antiserum could be washed free from serum, and then produced passive sensitization when injected into a guinea pig. His experiments, which exclude the possibility of antiserum having been transferred with the precipitate, could easily be explained by a dissociation of the precipitating antibody from the precipitate after injection. Since such precipitates were found by him both to transfer active as well as passive anaphylaxis, the supposition is that the antigen and antibody united in the precipitate may have dissociated and in this way produced both effects. The further experiments of Weil in which he removed the precipitating function of a serum by heating at 70° (or by extraction of the antibody from the precipitate with sodium carbonate), without removing its sensitizing functions, are not in our opinion contradictory to the general identification of these antibodies, since it is well known that the actual floculating properties of an antiserum may be physically interfered with by heating, and that an alkalinity above a Ph of 8.5 to 9 definitely prevents agglutination and precipitation.

Symptoms of Anaphylactic Shock.—If a properly sensitized guinea pig receives a second injection of an antigen after a suitable incubation time a very characteristic train of symptoms ensues. There is usually a short preliminary period—lasting either a fraction of a minute or several minutes according to the violence of the reaction and the mode of administration—during which the pig appears normal. At the end of this time the animal will grow restless and

[72] Weil, Richard. *Jour. Immunol.*, 1, 1916, p. 19.

uneasy, and will usually rub its nose with its forepaws. It may sneeze and occasionally emit short coughing sounds. At the same time an increased rapidity of respiration is noticeable and the fur will appear ruffled. In light cases the animals may remain in this condition, with further irregularity and difficulty of respiration, possible discharges of urine and feces; then gradual slow recovery may set in, with complete return to normal in from 30 minutes to several hours. In more severe cases these preliminary stages are rapidly followed by great apparent weakness. The animals fall to the side, the legs and trunk muscles twitch irregularly, and the respiration becomes slow and shallow; the thorax never entirely contracts, but remains in a more or less expanded condition. The very evident dyspnea is of an inspiratory character. The excursions of the lung itself seem to grow shallower and shallower in spite of apparent strong inspiratory efforts—the volume of the thorax and lung remaining in the expanded condition. At this stage evidences of motor irritation may appear, in that the animal may arise and attempt to run. More often, however, in this phase general convulsions set in, often several times repeated, and in these the animals usually die.

On the other hand, after cessation of convulsions they may lie perfectly still on the side as though paralyzed, the breathing becoming gradually slower and more shallow, finally ceasing entirely. The heart may continue to beat for a considerable time after the breathing has stopped.

If such an animal is immediately autopsied a very characteristic condition is found—to which, in the essentials, attention was first called by Gay and Southard.[73] They speak of finding "pulmonary emphysema as a constant feature at autopsy," and attribute the anaphylactic death in guinea pigs to cessation of respiration in the inspiratory phase under the influence of respiratory central intoxication.

The lungs of such guinea pigs after death are found distended and completely filling the thorax. They are usually pale and bloodless and do not collapse as the pleuræ are opened. On microscopic examination the alveoli are seen to be distended and small hemorrhages may appear upon the serous surfaces. According to Gay and Southard, furthermore, histological study of the other organs shows also hemorrhages in the brain, stomach, heart, cecum, and spleen—more rarely in other organs—and there are local fatty changes in the capillary endothelium which they regard as causatively related to the hemorrhages.

That the respiratory symptoms are the most striking feature of the clinical picture of guinea pig anaphylaxis had, as a matter of fact, been noticed by Rosenau and Anderson. A detailed physiologi-

[73] Gay and Southard. *Jour. Med. Res.*, Vol. 16, 1907; Vols. 18 and 19, 1908.

cal study of the mechanism of the respiratory death in these cases was first made, however, by Auer and Lewis [74] in 1909.

These investigators showed that, during the later respiratory symptoms, little or no air enters the lungs, although the animal makes violent respiratory efforts. This is due, as they found, to a tetanic contraction of the small bronchioles, which practically occludes the air passages. That the origin of this contraction is not, as previously supposed, of central origin, but is referable to peripheral cause, they proved by showing that the same phenomena occur in the guinea pigs even after the cord and medulla have been destroyed and the vagi divided. In such cases, of course, with the cord and medulla destroyed, artificial respiration had to be done, and when the symptoms set in it was found that the lungs could no longer be expanded by the same force of artificial respiration which before this had been sufficient.

They showed also that the non-collapsible expansion of the lungs after death was due to imprisonment of the air in the alveoli by the contracted musculature of the small bronchioles, and further confirmed their opinion of the peripheral origin of this contraction by the important discovery that atropin will markedly protect, often preventing death or hastening recovery. It is noteworthy, too, that Auer and Lewis speak of occasionally finding slight pulmonary edema, a feature which Biedl and Kraus consider incompatible with true anaphylaxis.

Anderson and Schultz,[75] who have confirmed much of the work of Auer and Lewis, find that not only atropin will prevent asphyxiation in these cases, but methane, chloral hydrate, adrenalin, and pure oxygen will exert a similar effect. The animals may be saved from suffocation in this way, but may nevertheless die, probably as the result of lowered blood pressure.

The observations of Auer and Lewis have been further confirmed especially by Biedl and Kraus,[76] who regard it as well established that anaphylactic death in guinea pigs is caused primarily by suffocation, due to tetanic spasms of the musculature of the small bronchi. These spasms are not of central origin, but are peripherally initiated, possibly by direct action upon the smooth muscle itself. The fact that atropin is not effective in preventing death in all severe cases is no argument against this, since such an effect would naturally depend upon the relation between the amount of atropin given and the severity of the attack. In this connection the studies that have been made upon the irritability of smooth muscle fibers in normal and in

[74] Auer and Lewis. *Jour. A. M. A.*, Vol. 53, p. 458, 1909; *Jour. Exp. Med.*, Vol. 12, 1910.

[75] Anderson and Schultz. *Proc. Soc. Exp. Biol. and Med.*, 7, 1909, p. 32.

[76] Biedl und Kraus. *Zeitschr. f. Immunitätsforschung*, Vol. 7, 1910; *Centralbl. f. Physiol.*, 1910; *Wien. klin. Woch.*, No. 11, 1910.

sensitized animals are of great interest. Schultz,[77] following out an observation made by Rosenau and Anderson, studied the intestinal muscle of normal sensitized guinea pigs excised and suspended in Howell's solution. In this way he showed that during the period of hypersusceptibility the smooth muscle is abnormally sensitive to treatment with the antigen. The contraction which normally occurs in smooth muscles under the influence of serum is markedly augmented if the preparations are taken from sensitized animals.

In addition to these predominant features of the anaphylactic symptomatology in guinea pigs, there are a number of secondary reactions which, though less prominent, are nevertheless of considerable interest and theoretical importance. The conditions in the circulation are probably, to a great extent, dependent upon the respiratory condition, and the fall of blood pressure in guinea pigs is regarded by some investigators as merely a secondary manifestation just preceding death. The fall of temperature first described by H. Pfeiffer,[78] however, seems to be an occurrence which, though standing in no causative relation to the symptoms as a whole, is so constant and well marked that it has been taken by a number of workers as one of the necessary criteria for the characterization of the anaphylactic condition.

There is, indeed, an almost regular drop of several degrees in the rectal temperature, and a close observation of this may be of much aid in determining the occurrence of mild reactions, when other symptoms of shock are not strongly marked. Pfeiffer[79] himself goes so far as to claim that by this symptom alone delicate anaphylactic reactions may be determined when all other symptoms are lacking.

Friedberger,[80] too, has found the sudden drop of temperature a very regular occurrence, and has employed this method of study for the analysis of the intensity of anaphylactic shock. He calls attention to the apparent difference between infection and anaphylaxis in this respect in that in the former there is fever, in the latter there is depression of body heat; but, at the same time, he points out that this discrepancy is an apparent one only, and determined by quantitative differences, for when he treated sensitized animals with varying doses of antigen he found that quantities which produced other anaphylactic symptoms of noticeable degree would regularly depress the temperature as Pfeiffer had shown. It was possible, however, to determine a minimal dose necessary for temperature reduction. Quantities just below this left the temperature un-

[77] Schultz. *Jour. Pharm. and Exp. Therap.*, 1, 1910; 2, 1910.
[78] H. Pfeiffer. *Wien. klin. Woch.*, No. 1, 1909.
[79] Pfeiffer u. Mita. *Zeitschr. f. Immunitätsforschung*, Vol. 4, 1910.
[80] Friedberger. *Deutsche med. Woch.*, No. 11, 1911. Friedberger und Mita. *Zeitschr. f. Immunitätsforschung*, Vol. 10, 1911.

changed, and still smaller quantities produced fever or even increased the temperature. This fact is extremely significant in that, as we shall see, it has an important bearing upon views which interpret bacterial infection as a series of anaphylactic poisonings, the multiplying bacteria furnishing the constant supply of minute amounts of antigen. This thought, indeed, based also on the study of temperature curves in animals, was expressed by Vaughan [81] as early as 1909, and was developed by him with Cumming and Wright [82] in an extensive study upon what he called "protein fever." It was shown in these experiments that continued fever, not unlike that of infectious diseases, could be produced in rabbits by repeated subcutaneous injections of primarily harmless substances, such as egg white and vegetable proteins. The conditions observed and the conclusions drawn from them in this work, as well as in the similar investigations of other workers, were clearly foreseen by Vaughan in his early investigations on proteid split-products studies, which we will find occasion to discuss in a later section.

The rigidity of the diagnostic value of the temperature relations for anaphylactic shock in particular, as advanced by Pfeiffer, was somewhat weakened by Ranzi's [83] observations that foreign serum may produce temperature depression when injected into perfectly normal animals and that, injected into sensitized animals, the same reaction may follow if other proteins than the original antigen were administered.

Although these objections of Ranzi are perfectly just, yet there is such a marked quantitative difference between the reaction in normal and in sensitized animals that, in principle, Pfeiffer's claim is not invalidated. Friedberger [84] very logically remarks that, after all, the phenomena of sensitization as well as those of immunity are merely an exaggeration of normal physiological conditions, and in experiment he has shown that, whereas noticeable depressions of temperature will follow in the normal animal only upon quantities of antigen exceeding 0.5 c. c., the temperature of the sensitized animal may be depressed by amounts as small as 0.0005 c. c.

Apart from the symptoms so far discussed, there are other less apparent characteristics of anaphylaxis in guinea pigs, all of which, however, possess considerable importance theoretically. The most significant of these is the reduction in the amount of alexin or complement, first noticed by Sleeswijk,[85] which occurs after the injection of the second or toxogenic dose—during the development of shock.

[81] Vaughan. *Zeitschr. f. Immunitätsforschung,* Vol. 1, 1909.

[82] Vaughan, Cumming, and Wright. *Zeitschr. f. Immunitätsforschung,* Vol 9, 1911.

[83] Ranzi. *Zeitschr. f. Immunitätsforschung,* Vol. 2, 1909; *Wien. klin. Woch.,* No. 40, 1909.

[84] Friedberger u. Mita. *Loc. cit.*

[85] Sleeswijk. *Zeitschr. f. Immunitätsforschung,* Vol. 2, 1909.

This phenomenon is so closely interwoven with the later theoretical aspects of anaphylaxis that we will defer its discussion until we have completed a more general survey of the field.

In guinea pigs, as in dogs, Friedberger and others have also seen a lowered coagulability of the blood and a temporary diminution of the polynuclear leukocytes (leukopenia) during shock.

Differences Between Physiological Reactions of Guinea Pigs and other Animals.—We have described the occurrences in guinea pigs at some length because protein anaphylaxis has been most thoroughly studied in these animals. We have already seen, however, that in discussing active sensitization, there are marked differences in the anaphylactic phenomena as they occur in different species of animals. The physiological reactions noted in guinea pigs, therefore, as a result of anaphylactic insult, cannot, indiscriminately, be applied to other animals.

In sensitized *rabbits* the injection of a further dose of antigen is usually followed, after a short but definite incubation time, by great weakness with, often, discharge of urine and feces. The animals sink down until the abdomen touches the ground, the legs are stretched out weakly but not paralyzed, and the head may drop forward or to one side. After this, the animal may gradually fall upon its side and lie motionless except for labored and irregular breathing and occasional twitching of the legs and head. Sometimes this gradual relaxation may be interrupted by a sudden motor irritation, the rabbit suddenly getting up and running a short distance but soon falling down again apparently from a sudden return of the muscular weakness. During these running spells it seems as though there was no sense of direction or purpose—the animals running into obstructions or off tables as the case may be. During this period general convulsions and a drawing back of the head by a tetanic spasm of the muscles of the neck are not uncommon. Death may occur within a few minutes, or it may follow a gradually increasing weakness in the course of several hours. The fall of blood pressure here seems to be purely secondary to the general failure of all the functions.

Unlike guinea pigs, rabbits do not show the distention of the lungs found in guinea pigs. Characteristic, however, is the distention of the right side of the heart and, as Auer [86] first noted, there is a definite pathological change in the muscles of the right ventricle which he believed was directly connected with anaphylactic death. This supposition has been recently confirmed by an important observation by Coca [87] who perfused the vascular pulmonary system of normal rabbits and of rabbits dead of anaphylactic shock. He

[86] Auer. *Centralbl. f. Physiol.*, 24, 1911, p. 957.

[87] Coca. *Proc. Soc. Exper. Bio. & Med.*, 16, 1919, p. 47. Coca. "Anaphylaxis," Tice's Modern Medicine.

found that while a pressure of 10 cm. will easily perfuse the normal pulmonary vascular system of the rabbit, a similar experiment on rabbits that have just died of anaphylactic shock failed, even under a pressure of as much as 90 cm. When he cut the lung near the pleural surface in such rabbits, while this pressure was being applied, no fluid oozed through, proving that the obstruction is on the arterial side of the pulmonary circulation. He justly concludes that death in the anaphylactic rabbit is probably due to a tonic spasm of the muscular coat of the pulmonary arterioles. Coca suggests that a similar spasm in the systemic arterioles may take place in the anaphylactic rabbits, and that such a localization of the anaphylactic reaction in the arterial walls may account for the local manifestation spoken of above as the Arthus phenomenon which is peculiar to rabbits so far, and has not yet been satisfactorily explained.

Anaphylaxis in *dogs* has been very extensively studied, especially by Biedl and Kraus,[88] and by Pearce and Eisenbrey.[89] The symptoms in dogs are characterized by a rapid progressive fall in the blood pressure, followed by the symptoms of cerebral anemia. Anaphylactic dogs, after injection, will at first grow restless, vomit, and pass urine and feces. They then grow rapidly weak, fall to the ground, and continue to twitch and vomit and the respiration becomes labored and irregular. There is general weakness of the muscles, but no paralysis. The marked, constant, and characteristic feature of the condition in these animals is the fall of blood pressure. There is also a lessened coagulability of the blood, much more strongly developed than in guinea pigs and rabbits.

According to Biedl and Kraus this may amount to almost a prevention of the coagulation in anaphylactic dogs.

As in other animals the blood picture is changed in that there is a falling off of the total number of leukocytes with a relative diminution of polynuclear cells.

Quantitative measurements by Calvary,[90] moreover, have shown that anaphylaxis in dogs is accompanied by a marked increase of the lymph flow (7 times the amount observed in normal dogs in the same time) and, by controlling the blood pressure with barium chlorid, that this lymphagogue action is not directly dependent upon the low pressure. This observation is of especial interest in connection with the similarity of anaphylaxis to peptone poisoning in which Heidenheim [91] noticed a similar increase of the lymph.

Pearce and Eisenbrey found, at autopsy of dogs dead of anaphylactic shock, subserous petechial hemorrhages in the rectum and gall

[88] Biedl and Kraus. *Loc. cit.;* also in "Kraus u. Levaditi Handbuch," Ergänzungsband 1.

[89] Pearce and Eisenbrey. *Proc. Soc. Exp. Biol. and Med.*, 7, 1909, p. 30; *Trans. Cong. Am. Phys. and Surg.*, Vol. 8, 1910.

[90] Calvary. *Münch. med. Woch.*, No. 13, 1911.

[91] Heidenheim. *Pflüger's Archiv,* 49, 1891.

bladder, hemorrhagic spots on the gastric and duodenal mucosa, and in the colon. According to these workers, in agreement with Biedl and Kraus, the fall of blood pressure is not due to central causes but depends upon influences exerted upon the peripheral vasomotor system. Biedl and Kraus believe that this action is exerted upon the muscle cells themselves rather than on the nerve endings. They admit the inconclusiveness of their experimental data, but take the above standpoint because of the fact that adrenalin, which acts by stimulation of the vasomotor nerve endings particularly, does not raise the low pressure in dogs during anaphylaxis while barium chlorid, which acts upon the smooth muscle fibers themselves, strongly raises the blood pressure in such animals. Pearce and Eisenbrey are inclined to believe that the action is chiefly upon the nerve endings, though both factors, nerve and muscle, may be involved. They worked with apocodein, a substance which, in large doses, paralyzes the vasomotor nerve terminals.[92]

When a sensitized dog was treated with apocodein and the antigen then injected, no further drop of pressure was obtained. Apparently a paralysis of the vasomotor nerve endings had removed the point of attack upon which the anaphylactic poison could act.

In addition to the symptoms already enumerated Weichhardt and Schittenhelm [93] claim that anaphylaxis in dogs is invariably accompanied by a severe local reaction in the gut. The intestinal mucosa is swollen and contains miliary hemorrhages and the lumen is often filled with a mucus mixed with blood. In the further analysis of the anaphylactic reaction in dogs, Manwaring [94] has recently reported observations of great interest. He investigated the participation in anaphylactic shock of the various organs and determined that shock did not occur when the abdominal vessels were ligated just above the diaphragm. In further localizing the source of shock he found that exclusion of the spleen, stomach, kidneys, suprarenals, and ovaries from the circulation had no effect upon the occurrence of anaphylactic shock. However, when he operated in such a way that the liver was thrown out of circulation, none of the seven dogs that he used reacted with anaphylactic shock to the injection of serum. He concludes from this that the liver is directly responsible in some way for the production of anaphylaxis. The intestines, too, were found, by a similar procedure, to take part, though to a less important extent than the liver.

It would seem, in view of a number of confirmations of Manwaring's work, that the great importance of the liver in anaphylactic dogs cannot be questioned. Richard Weil, who also investigated these conditions, believed that most of the circulatory disturbances in the

[92] Brodie and Dixon. *Jour. Phys.*, 30, 1904.
[93] Weichhardt and Schittenhelm. *Deutsche med. Woch.*, 19, 1911.
[94] Manwaring. *Zeitschr. f. Immun.*, Vol. 18, 1911.

anaphylactic dog could be attributed to obstruction in the portal circulation.

Recognizing as fairly well established that in the three animals most thoroughly studied in anaphylaxis, namely, the guinea pig, rabbit and dog, physiological reactions and localization in organs vary distinctly, many attempts have been made to explain such differences. In guinea pigs, as we have seen, the cause of death resides very largely in the musculature of the bronchioles, in rabbits a similar cause is found in the circulation of the lung, and in dogs the hepatic and splenic circulations seem to be particularly involved. A most ingenious and plausible explanation is suggested in Wells' review as the result of anatomical studies by a number of different workers. He states that histological study of guinea pigs has shown an astonishing development of the bronchial musculature, the finer bronchioles consisting almost entirely of muscular tubes. In rabbits "the pulmonary arteries present a remarkable degree of muscular development analogous to that of the guinea pig bronchioles." As far as dogs are concerned, he quotes Simonds [95] as stating that the walls of the hepatic veins of the dogs are different from those of any other animal, again, in showing a very high development of musculature. And, Wells concludes, it is at least a reasonable hypothesis that the differences in these species, in reaction to one and the same mechanism, depend upon fortuitous differences in the distribution of non-striated muscle. Interesting in connection with Wells' suggestion, is the fact that Huber and Koessler [96] have shown that asthmatic individuals show a hypertrophy of the musculature of the bronchioles which transforms them histologically into a condition not unlike that normally prevailing in the guinea pig, and it is well known that in such individuals severe attacks of an asthmatic nature can be elicited on serum injection. As a matter of fact, it is in individuals of this sort that one avoids or exercises unusual care in connection with protein injections.

Other animals than those mentioned have been little used for anaphylactic experiment. Observations incidental to other work, however, have shown that horses and goats are particularly sensitive. In goats the writer has observed both serum and bacterial anaphylaxis, and the symptoms here were those of general trembling, weakness, labored respiration, and involuntary evacuation of urine.

The lower *monkeys* are exceedingly difficult to sensitize. Our own experiments on this subject have already been cited. When, after repeated injection, sensitization is accomplished, the symptoms are usually mild, and not unlike those prevailing in human beings, in fact, we have cited a single case in which œdema of the face and a picture somewhat similar to serum sickness in man resulted.

[95] Simonds. *Jour. A. M. A.*, 73, 1919, p. 1437.
[96] Huber and Koessler. *Arch. Int. Med.*, 1921.

Mice can be sensitized but not as easily as guinea pigs. The best method consists in giving several relatively large preliminary doses a few days apart. Anaphylactic reactions in *man* will be discussed in a subsequent section.

Rats are refractory to sensitization. Longcope's work with isolated rat uteri was entirely negative. F. and J. T. Parker in our laboratory have obtained mild uterin reactions, but so far no uterin response.

In the discussion of the anaphylactic reaction given above, we have dealt chiefly with the very severe symptoms of acute shock. It must not be forgotten, however, that these conditions are the manifestations of a very acute and profound injury, and the localizations in various organs with which we have dealt are chiefly those which are responsible for death, perhaps to a large extent, or entirely, because these particular sites of injury affect the tissues upon which the immediate continuation of life depended. It is not at all out of question, however, that many other parts of the body may suffer, although their injury might have no immediately noticeable effects, but expresses itself, eventually, especially if the insult is repeated, in degenerations and chronic disease. Chiray [97] and Longcope [98] have attempted to approach this problem by repeated administrations of protein in moderate doses to rabbits and investigation of subsequent changes in the kidney. The lesions found by Longcope were interpreted by him as perhaps signifying a subacute anaphylactic injury. This conclusion, however, has been questioned, since then, by a number of workers who believe that similar lesions are too frequently found in untreated animals to permit definite assumption that the repeated protein injections had caused them. Boughton [99] has made observations similar to those of Longcope, and though the question must remain an open one for perhaps some time to come, it is, nevertheless, reasonable to suppose that, whether or not these particular experiments of Longcope and of Boughton can be accepted as proof, the likelihood is strong that repeated anaphylactic reactions of whatever severity, may cause injury to many organs in which they cannot be immediately observed.

Desensitization or Antianaphylaxis.—In properly sensitized animals the result of a sufficient dose of antigen, given at the proper time, is very often death. When the time and quantity are so chosen that instead of death there is merely a more or less severe anaphylactic shock, the animals are immediately thereafter in a *refractory condition*. That is, they are no longer sensitive to further injections of the antigen. This observation was made by Otto and by Rosenau and Anderson in their pioneer investigations, was confirmed by Gay and Southard, and was subsequently very thoroughly studied by

[97] Chiray. Thèse de Paris, 1906, cited from Longcope, *loc. cit.*
[98] Longcope. *Jour. Exper. Med.*, 22, 1915, p. 793.
[99] Boughton. *Jour. Immunol.*, 4, 1919, p. 213.

Besredka and Steinhardt.[100] The last-named workers named this refractory or immune condition *"antianaphylaxis."* There is obviously a great deal of both practical and theoretical significance in this fact, and methods were sought by which such an antianaphylactic state might be induced in sensitized animals without subjecting them to the dangers of actual shock. It was found that this could be accomplished in a number of ways. According to Besredka and Steinhardt the injection of moderate quantities of the antigen at a time just preceding the development of hypersusceptibility, in the preanaphylactic period, will render them refractory to later injections. This preventive administration, however, must be given during the later days of the anaphylactic incubation time. If given too soon after the first injection it does not prevent eventual sensitization, though it may occasionally delay its development, acting then simply as though a larger dose had been given in the first place. Thus if antigen is given by a method of introduction and in a quantity which would justify us in expecting hypersusceptibility to be developed at the end of 12 days, we can render the animal "antianaphylactic" by a second administration given, say, on the 8th, 9th, or 10th day. If we give it on the 2d, 3d, or 4th day after the first injection, it is very likely that sensitization will proceed nevertheless. Rosenau and Anderson have also investigated the repeated injection of antigen during the incubation time, and their results would also seem to emphasize the necessity of making the preventive injection close to the time at which hypersusceptibility may be expected. If quantities of 2 c. c. were injected 10 times in the course of 17 days, and 15 to 17 days thereafter 6 c. c. of horse serum were given, the animals showed symptoms proving that antianaphylaxis was but partial. If amounts of 0.001 c. c. were given 5 times in a period of 8 days, and the animals were tested 23 days later, death often ensued. It is also possible, as a number of investigators have shown, to produce the antianaphylactic state by the injection of sublethal doses, even after the time has set in at which the animals are hypersusceptible. This method can be carried out successfully according to Besredka by injecting very small amounts into the brain (1/50 to 1/400 of a cubic centimeter). Within a few hours after such an injection the animals may withstand an otherwise fatal dose with slight or no symptoms; although it is generally stated that intraperitoneal injections, carried out after hypersusceptibility has set in, must be of considerable quantity (large enough to cause symptoms) in order to induce antianaphylaxis. Besredka [101] states, in a recent résumé, that 1/50 to 1/100 cubic centimeter injected intraperitoneally and giving "practically no symptoms" in a sensitized guinea pig, after the anaphylactic state has set in, may render the animal entirely refractory after 5 hours.

[100] Besredka and Steinhardt. *Loc. cit.*
[101] Besredka in "Kraus u. Levaditi Handbuch," Ergänzungsband I.

On the other hand, Rosenau and Anderson,[102] working with *subcutaneous* injection, obtained results which differ considerably from those of Besredka. They sensitized a series of guinea pigs with mixtures of toxin and antitoxin, and 48 days later, at a time when the animals were hypersusceptible, gave 20 subcutaneous injections of 0.001 c. c. daily. Two days after the last injection, 0.2 c. c. of horse serum was given intracerebrally, and all of the animals showed symptoms, and many of them died. They conclude, therefore, that the repeated injection of small amounts of antigen into sensitized animals has no appreciable effect. The same worker has shown by experiment that the introduction of large amounts of antigen into the previously cleansed rectum of sensitive animals is entirely without danger and will produce an antianaphylaxis, which becomes evident after 12 hours. This is probably dependent upon the very slow penetration of small amounts of antigen into the circulation from the gut, and has, therefore, an effect similar to the repeated injection of small amounts directly, or the very slow and gradual method of intravenous injection advocated by Friedberger for the prevention of serum sickness in man. This phase of the subject is considered in greater detail in a subsequent discussion of serum sickness.

Antianaphylaxis produced in this way is specific,[103] although, as we shall see, there are other methods by which it is claimed that a nonspecific antianaphylaxis can be produced.

Such specificity readily appears from the fact that if an animal is sensitized, either actively or passively, to two or more antigens, separate desensitization can be accomplished with each individual antigen. Yet, as we shall see, in such experiments there is a certain degree of non-specific protection as a consequence of the first desensitization, a matter to which we will recur presently.

It is plain, therefore, that since it is pretty well established that antianaphylaxis means merely a desensitization by a partial or complete saturation with antigen of the antibodies present in the sensitized animal, quantitative relations must be extremely important in this reaction. For, if a dose of antigen is injected into a highly sensitive animal which is insufficient completely to saturate the antibodies, partial desensitization only could be expected. And, conversely, probably the most thoroughly desensitized animal is one that has recovered from a dose of antigen, almost but not quite fatal. Moreover, since the suddenness of union of antigen with antibody seems to determine the severity of the reaction, any method in which a sufficient amount of antigen can be introduced in a manner so gradual that the union must needs be slow, may serve to desensitize

[102] Rosenau and Anderson. *Loc. cit., U. S. Pub. Health and M. H. S. Hyg. Lab. Bull.* 45, 1908.

[103] Pfeiffer has recorded an exception to this in that he claims to have rendered a horse-serum-sensitive animal refractory by an injection of swine serum.

without severe symptoms. This, then, should be the principle in all methods of desensitization. Besredka, as we have seen, attempted to accomplish this by the repetition of minute doses, whereas, Friedberger and Mita [104] accomplished the same purpose by administering the antigen very slowly, by a gravity feed apparatus and in a dilution; we have already mentioned attempts to desensitize through the gut, a method based on the same principle.

This mechanism of desensitization explains why the refractory condition should supervene almost immediately after the injection of the antigen, whether or not shock has taken place. Also, such an understanding will indicate that there is no fundamental difference in the desensitization of actively or passively sensitized animals.

Although it is somewhat anticipating the theoretical considerations to be taken up in the next chapter, we may mention in this place that the now generally accepted conception that the anaphylactic reaction takes place largely between the antigen and antibodies which are in some way in union with tissue cells, has led to the very natural supposition that perhaps shock can be prevented by supplying a sufficient concentration of homologous antibodies to the circulation just before administering the shock dose of antigen. The reasoning here is identical with that on which we explained the protective value of antitoxin, in that the circulating antibody, by uniting with the antigen, prevents the latter from reaching the susceptible cells. And for our present purposes we may conceive the sensitized cell as readily amenable to injury by the protein antigen, as the normal cell would be to toxins of the diphtheria or tetanus bacillus. Weil, as far as we know, was the first to subject these conditions to careful analysis, and, indeed, found that he could protect actively or passively sensitized guinea pigs by introducing into the blood large amounts of homologous immune body previous to the shock injection. Although the theoretical conception was thus upheld, he found that unexpectedly large amounts of antibody were needed for this purpose, a fact which he explains by "an increased avidity of the 'anchored' antibodies, as compared with the free antibodies, for antigen." Our own opinion which differs only in the principle of mechanism from that of Weil, is based upon the observation noted in another chapter that antigen and antibody in the circulation are prevented from uniting with any degree of speed, probably by the colloidal protective action of other serum constituents; and that, in consequence, a considerable excess of antibody in the circulation is necessary in order to render an experiment of this kind successful. Nevertheless, from a practical point of view, an excess of antibodies in the blood might be a safeguard of considerable value, when the antigen is at the same time injected slowly and with caution. Some of the other points involved in these very interesting researches of Weil will be dis-

[104] Friedberger and Mita. *Zeitschr. f. Immunität.*, 10, 1911.

cussed in the following chapters in which we will deal with theories of anaphylaxis.

Of practical importance is the *duration of antianaphylaxis.* As one would expect, a dose of antigen which renders a sensitive animal refractory, would, after the first reaction, act merely as a further antibody producing stimulus. In consequence, then, one would expect the animal to return to the sensitive condition after a relatively brief period. In guinea pigs, according to a number of observers, the refractory condition may last two weeks or longer. In rabbits, according to Scott [105] the return to the sensitive condition is very rapid, perhaps because of the energetic antibody production observed in these animals.

Non-Specific Desensitization.—The complete or partial desensitization of a sensitive animal by injection of the antigen appears obviously to be due to the partial or complete saturation of the sessile antibodies by the antigen. Less clear is the unquestionable fact that partial desensitization can be non-specifically accomplished in a number of ways. Dale made the observation that the uterus of a guinea pig sensitized against two different antigens and then desensitized completely to one of them, loses a certain amount of sensitiveness to the other antigen subsequently instilled into the bath. Biedl and Kraus,[106] moreover, in their studies of peptone shock in dogs, reported that the injection of peptone into a hypersensitive dog produced a considerable resistance against subsequent injection of the antigen. Similar conditions have been noted in connection with certain poisons which we shall speak of later as "anaphylatoxins" as prepared by Friedberger. These, as we shall see, are toxic products obtained in various ways from bacteria, agar suspensions, etc., which give, on injection into guinea pigs, symptoms simulating anaphylactic shock. Bessau [107] passively sensitized guinea pigs with 1 c. c. of anti-horse serum intraperitoneally, and on the following day injected them intravenously with 1 c. c. of horse serum. He gauged his dose so that the animals should have severe shock but survive. One or two days later he injected the amount of typhoid anaphylatoxin which was fatal for normal pigs, and found that those which had been treated as described were now able to withstand the anaphylatoxin. These experiments of Bessau would indicate that antianaphylaxis was to a certain extent due to tolerance to the poison, and that it was non-specific. Friedberger,[108] together with Szymanowski, Kumagai, Odaira and Lura, later studied this problem and came to the conclusion that antianaphylaxis is strictly specific, depending, as Friedberger had suggested, upon the diminution of specific antibodies rather than upon tolerance to the poison. They

[105] Scott. *Jour. Path. Bact.*, XIV, 1909, and XV, 1910.

[106] Biedl and Kraus. *Wien. Kl. Woch.*, No. 11, 1909.

[107] Bessau. *Centralbl. f. Bakt.*, 60, 1911.

[108] Friedberger and Szymanowski, etc. *Zeitschr. f. Immunitäts.*, 14, 1912.

claimed that animals that had been sensitized and then had survived the "shock" dose of homologous protein showed no tolerance for anaphylatoxin, and that animals that had been treated with the sublethal dose of anaphylatoxin are, for 24 hours, as sensitive to anaphylatoxin, however prepared, as are normal animals. Recent studies along the same lines by Zinsser and Dwyer [109] have yielded results differing from these conclusions. Working with typhoid anaphylatoxin they found that guinea pigs treated with a sublethal dose of anaphylatoxin develop a tolerance which enabled them to resist 1½ to 2 units of poison, the tolerance developing within three days and lasting, to a slight degree, for as long as two months. It seemed to them that animals treated with a second dose of anaphylatoxin within 24 hours after the first, if the results of this first injection have been severe, as they usually are, still weak and generally depressed in vitality so that a developed tolerance may be clouded by this condition. The tolerance did not seem to be strictly specific in that typhoid anaphylatoxin seemed to produce a moderate tolerance to prodigiosus anaphylatoxin.

It would seem, therefore, that in antianaphylaxis we might have two very important elements. The one strictly specific depends upon the depletion of antigen from the body, a true "desensitization." The other non-specific, and probably of secondary importance since so far it has not been shown to any very powerful degree, consists of the development of tolerance by the body cells for the anaphylactic poison.

Of similar bearing are the observations of Vaughan [110] who, working with his toxic split products of the Colon bacillus protein, found that repeated injections into guinea pigs produced a tolerance which permitted the animals to resist about double the fatal dose, but did not protect against larger multiples.

The preventive influence of atropin in anaphylaxis has already been noted in connection with the work of Auer and Lewis. Besredka, as we have also seen before, claims to have prevented fatal shock by ether anesthesia on the theory that most of the reactions took place in the central nervous system. It is more than likely that Besredka slightly underdosed his animals, and by the ether anesthesia merely prevented convulsive symptoms. Banzhaf and Famulener [111] prevented shock by large doses of chloral hydrate. Rosenau and Anderson,[112] carrying on similar investigations with urethan paraldehyde, chloral hydrate and magnesium sulphate, came to the conclusion that

[109] Zinsser and Dwyer. Reported at the meeting of Am. Ass. of Path. & Bact., Toronto, April, 1914.

[110] Vaughan. "Protein Split Products, etc.," Lea & Febiger, Phila., 1913, p. 139.

[111] Banzhaf and Famulener. *Stud. N. Y. Dept. Health, Labor.*, 1908, p. 107.

[112] Rosenau and Anderson. *Jour. Med. Res.*, No. 21, N. S. 16, 1909.

none of these drugs had any noticeable effect on anaphylactic guinea pigs. The interesting observation of Friedberger [113] that injections of moderate amounts of hypertonic salt solution would diminish the severity of anaphylactic shock was explained by him on the basis that hypertonic salt prevented complement or alexin action, and thereby diminished the production of anaphylatoxin, a poison which, we shall see, he believes is produced by the action of alexin on the antigen antibody complex, and is responsible for anaphylaxis. Zinsser, Lieb and Dwyer,[114] however, were able to show that the hypertonic salt solution probably acts by diminishing the irritability of smooth muscle in the animal body and thereby reduces the severity of the spasms in the bronchioles and other smooth muscle tissues of the guinea pig, preventing death in otherwise fatal experiments.

[113] Friedberger and Hartoch. *Zeitschr. f. Imm.*, Vol. II, 1909.
[114] Zinsser, Lieb and Dwyer. *Jour. Exper. Biol. and Med.*, 12, 1915.

CHAPTER XVII

ANAPHYLAXIS (*Continued*)

In view of the rapidity with which the surprising facts of protein hypersusceptibility were uncovered, it is but natural that many of the earlier theories conceived regarding the phenomena developed along lines which now seem fantastic. Yet, we must remember that there was much conflicting evidence about some of the fundamental facts, and it was some time before the recognition that an antigen-antibody reaction was basic to the occurrence, was firmly established. Among the most prominent of these earlier theories were those of Gay and Southard and of Besredka, and while these views have now been definitely discarded, the discussion to which they led, nevertheless, stimulated much interesting investigation.

These two theories were premised upon the view that the injected antigen (horse serum, egg white, etc.) contained two separate substances, one of them responsible for the sensitizing process, the other giving rise to shock on subsequent injection.

The views of Gay and Southard [1] are summarized in the following paragraphs given, as nearly as space permits, in their own words:

Increased susceptibility in the sensitized animal is due to the continued presence in the circulation of an unneutralized element of the antigen (in their case horse serum), which they call "anaphylactin," which acts as an irritant or stimulant to the body cells, and, in some way, causes them to assimilate over rapidly certain other elements of horse serum. These assimilated or toxic elements are the same as those eliminated without producing intoxication during the incubation period following the first dose. This over-assimilation after anaphylaxis is the cause of the intoxication.

Gay and Southard find much support for their contentions in the results of experiments done with the so-called "passive" transfer of hypersusceptibility. As mentioned above, hypersusceptibility may be transferred to a normal animal with the blood serum not only of a sensitive animal, but even more surely and effectually with that of a refractory, or "antianaphylactic," animal. They believe that such transfer is not "passive" but "active" sensitization, being accomplished by the transfer of "anaphylactin" to the normal animal. The refractory animal has received more horse serum than the merely sensitive one, since antianaphylaxis is produced by massive injections. Therefore its blood contains more anaphylactin and is consequently more active in transferring sensitiveness. The fact that a considerable incubation time is necessary in active sensitization they attribute to the gradual action of the anaphylactin.

[1] Gay and Southard. *Jour. Med. Res.*, Vol. 16, 1907; Vol. 18, 1908; Vol. 19, 1908.

In passive sensitization, therefore, they assumed a similar gradual irritation of the vulnerable cells by the anaphylactin and, as we have seen, obtained their reactions in animals so treated, usually 10 to 14 days after the sensitive serum had been given. This conception of the mechanism of passive anaphylaxis was, of course, rendered unlikely by the demonstrations by Friedemann, Otto, and others that shock could be elicited in passively sensitized animals within 24 hours or less after transfer of the anaphylactic serum.

To this, however, Gay and Southard [2] answer by implying that this greater speed of development of sensitiveness in the experiments of Otto is due to the larger doses used by him. They say "if the doses are sufficient it (transmitted sensitiveness) may be shown in a single day (Otto)." However, it is very likely that the sensitiveness noted by them in animals two weeks after the transference of anaphylactic serum was actually positive sensitization with antigen rests, entirely comparable to the usual "Theobald Smith" phenomenon.

Gay and Southard's definite objections to the possibility of an antigen-antibody reaction are found in the following arguments based on experimental observations:

1. Sensibility persists for a long time, antibodies disappear rapidly.
2. In the serum of animals sensitive to horse serum antibodies to this serum are not demonstrable by complement fixation.
3. Although sensitiveness can be transferred to a normal animal, nevertheless a definite period of incubation must elapse before the recipient becomes sensitive.

To the first of these arguments Besredka [3] objects by saying that, while it is true that sensitiveness persists for a long time, the power to transmit anaphylaxis passively disappears rapidly, as Otto, Richet, and others have shown.

The second contention is contradicted by the work of Nicolle and Abt.[4] But since these workers made their observations upon rabbits their experiments do not necessarily contradict those of Gay and Southard. This point at best is a difficult one to determine, especially as recent investigations have shown us that under certain circumstances antigen and antibody may be found side by side in the same serum without uniting and without therefore fixing alexin or complement.

The point of their third argument has been discussed above.

It is clear that Gay and Southard separate distinctly the substance in the antigen which sensitizes from that which exerts the toxic action on second injection.

Another theory which is based on such a separation of a sensitizing and shock-producing element in the original antigen is that of Besredka.

Besredka [5] assumes that in the injected antigen (serum) there are two separate substances. One of these, the *sensibilisinogen,* induces, during the time of incubation, a specific antibody (*sensibilisin*). This antibody remains in part attached to issue cells and in part circulates freely in the blood. The other substance in the antigen he calls "*antisensibilisin.*" This, at the second injection, reacts with the sensibilisin and anaphylactic shock results. The nature of the symptoms is explained by the fact that the antibody or sensibilisin is attached to cells of the central nervous system, and shock can

[2] Gay and Southard. *Jour. Med. Res.,* p. 427, 1908.
[3] Besredka. *Bull. de l'Inst. Past.,* 6, 1908, p. 826.
[4] Nicolle and Abt. *Ann. de l'Inst. Past.,* Vol. 22, p. 132, 1908.
[5] Besredka. *Loc. cit.*

result only when such attachment is present. Thus, in passive transference of sensitization, the property of hypersusceptibility is bestowed upon the normal animal by the sensibilisin or antibody present in the circulating blood, but the significance of this body for anaphylaxis is not in evidence until a connection with the central nervous system has been established.

There is much in Besredka's theory which is at variance with prevailing conceptions of biological phenomena of this category. The fact that an antigen should give rise to an antibody which reacts not with the substance that induced it, but with a third body, is quite out of keeping with experience.

However, it is clear that in both theories, that of Gay and Southard, as well as that of Besredka, the cardinal point is this separation in the antigen of two substances, a sensitizing and a toxic or shock-producing, and, since this forms the chief argument against an antigen-antibody conception of anaphylaxis, it will be necessary to examine the experimental evidence on which it is based.

Gay and Adler [6] attempted to show such a dual function of the original antigen by chemical methods. They report that, by fractional precipitation of horse serum with ammonium sulphate, the successive protein fractions obtained, as saturation is increased, are found to be less sensitizing and more toxic as more and more ammonium sulphate is added. The first fraction (euglobulins) obtained by 1/3 saturation is as sensitizing as whole serum and corresponds to anaphylactin, but is nontoxic when injected into sensitive animals. The last fraction, while distinctly less sensitizing than either the whole serum or the first fraction, is at least as toxic as the whole serum.

In these experiments, therefore, we have a strong argument in favor of the separate presence in an anaphylactic antigen of two bodies, the one sensitizing and the other toxogenic. However, this assertion has not been borne out by later work.

Pick and Yamanouchi,[7] whose extensive investigation cannot be fully reviewed here, were unable to obtain such a separation; in fact, they conclude that the same substances which sensitize are also toxic, and, working with a large variety of methods, find that both the sensitizing and toxogenic properties of proteins show no differences either in chemical condition or in resistance to chemical agents or heat.

The work of Pick and Yamanouchi, however, was done with rabbits and, therefore, as bearing on the theory of Gay and Southard, the work of Doerr and Russ [8] is more directly to the point. These workers using guinea pigs, and both horse and beef sera, obtained results which are practically diametrically opposed to those of Gay and Adler. They found that the euglobulins, obtained by 1/3 saturation with ammonium sulphate, are the most strongly sensitizing and, at the same time, the most toxic of the fractions of the sera. As saturation with the salt is increased, the proteins which come down decrease progressively and in parallelism, both as regards the power to sensitize and the faculty of exerting toxic action on second injection. The albumin, which finally comes out on total saturation, is devoid both of sensitizing and of toxic properties. Similar results were obtained by Doerr and Russ with the precipitation of serum proteins with CO_2.

The weight of evidence, therefore, seems to point against a chemical separation of the two functions in the antigen.

Besredka's contentions in favor of such a separation were based chiefly upon a difference in resistance to heat.

His experiments showed that the sensitizing properties of serum are not lost even if it is heated to 120° C., while the toxogenic powers are destroyed

[6] Gay and Adler. *Jour. Med. Res.*, Vol. 13, 1908.

[7] Pick and Yamanouchi. *Zeitschr. f. Immunitätsforschung,* Vol. 1, 1909.

[8] Doerr and Russ. *Zeitschr. f. Immunitätsforschung,* Vol. 2, 1909.

by much lower temperatures. The results of Besredka as to the differences in thermostability between the two properties have found confirmation by Kraus and Volk [9] and others, and there can be little doubt that the sensitizing function is extremely heat-resistant, since this has also been shown by Wells,[10] Rosenau and Anderson, and many others. However, researches by Doerr and Russ,[11] and notably by Wells, have shown that, though not *destroyed* by high temperatures, even moderate heating markedly diminishes the sensitizing function, and that larger doses have to be given as the temperature is increased; and since the smallest quantities of antigen necessary for inducing shock at the second injection must be anywhere from 100 to 1,000 times as large as the smallest sensitizing doses, it is quite likely that a combination of such conditions might stimulate an actual difference in heat resistance. In fact, this is the view expressed by Wells [12] and borne out by experiments carried out by Doerr and Russ.

Wells, too, confirms the identity of sensitizing and toxic substance by his experiments on the influence of tryptic digestion upon these properties of the antigen. He concludes that both sensitizing and intoxicating properties are attacked and slowly decrease as the coagulable protein disappears.

As to that aspect of Besredka's theory which deals with the indirect participation of the central nervous system, his arguments are based mainly on the fact that ether narcosis seemed, in his experiments, to prevent anaphylactic shock when animals were deeply anesthetized during the second injection, and also upon the regularity, severity, and speed with which anaphylactic symptoms follow injections directly into the brain. The former contention regarding narcotics cannot, by any means, be accepted as yet, since Rosenau and Anderson failed to confirm it and claim that ether narcosis merely masks the symptoms but does not prevent death. If we admit the beneficial effects of ether, moreover, it may well be that this is accomplished by relaxation of the bronchial spasms, known, since Auer and Lewis, to be the cause of death in guinea pigs, and the action of ether could hardly be utilized, therefore, to argue in favor of a central localization of the anaphylactic process.

That phase of the two theories so far mentioned, therefore, which depends upon the assumption of two separate substances in the original antigen does not seem established nor even sufficiently likely to warrant the formulation of a theory upon it.

The second premise is the necessary participation of the body cell, in that the reaction cannot take place unless the cells are rendered vulnerable by preliminary alteration. In Gay and Southard's theory this is assumed to occur by irritation exerted by the "anaphylactin," in Besredka's scheme it is attributed to the antisensibilisin which is attached to the nerve cells.

It is plain, therefore, that both Gay and Southard and Besredka admit a preliminary preparation of the cells of the body, and this is, as we shall see, an important factor in anaphylaxis, though not exactly in the sense of any of the observers named.

In contradistinction to the more complex theories of Gay and Besredka which we have just discussed, the early views of von Pirquet, of Rosenau and Anderson, and of most other observers who worked upon this subject, upheld the true antigen-antibody nature of the reaction with the antigen.

[9] Kraus and Volk. *Zeitschr. f. Immunitätsforschung,* Vol. 3, 1909.
[10] Wells. *Jour. Inf. Dis.,* Vol. 5, 1908.
[11] Doerr and Russ. *Loc. cit.*
[12] Wells. *Jour. Inf. Dis.,* Vol. 6, p. 521, 1909.

Site of the Reaction.—Subsequent investigation was for some time largely aimed at analysis of the pathological physiology of the reaction, and one of the most important points of controversy which developed was that which concerned the question of whether the harmful antigen-antibody union took place in the circulation or in or upon the cells of the body. With the antigen-antibody mechanism accepted, it is but natural that earlier views favored the probability that the harmful union of antigen and antibody took place in the circulating blood. It will be seen, however, that even in the earlier theories mentioned above, the possibility of a participation of the tissue cells in the reaction was hinted at. Friedberger [13] was probably the first to definitely suggest such a theory, although his subsequent work carried him farther away from the cellular ideas than any of the other workers. The cellular conception was very largely based from the beginning upon the observation that passive sensitization in guinea pigs regularly demanded an interval of six or more hours between the injection of the antibodies and the development of the hypersensitive condition. Exceptions to this and their interpretation we will discuss presently. However, the regularity with which this interval was necessary in guinea pigs, the animals most frequently used for experiments at that time, demanded an explanation. The simplest explanation presenting itself was to the effect that a certain length of time was necessary for attachment of the injected antibody to the cells, and that hypersensitiveness implied such "sessile receptors."

The idea, itself, was not new. Wassermann had first suggested it in an attempt to explain the peculiar hypersusceptibility of toxin possessed by some of Behring's toxin immunized animals. He assumed in such cases that the formation of antitoxin may, indeed, have been stimulated, but not to such a degree that the antitoxic substances had been to any extent distributed into the circulation. This he argued would leave the cell with a very much increased number of antitoxin complexes, capable of uniting with toxin, but still united to the cell plasma in some way. In consequence, the cell would be excessively vulnerable during the stage just preceding the concentration of the antitoxin in the circulation. As Behring expressed it, in the case of antitoxin, the very substance which when free in the circulation has protective functions because of its ability to unite with the toxin and deflect it from the cells, may represent an actual cause for increased injury when still attached to the cell.[14] The conception is quite plain and reasonable if one follows closely Ehrlich's point of view, and it is mentioned here chiefly because it was upon this reasoning that Friedberger first based his idea that the

[13] Friedberger. *Zeitschr. f. Immunitätsforschung*, Vol. 2, 1909, p. 208.
[14] See discussion of Side Chain Theory on p. 143, etc.

anaphylactic reaction might be a sort of cellular precipitin phenomenon.

Friedberger, however, because of a new turn taken by his investigations at this time, soon abandoned the cellular point of view, and in the course of subsequent development, two definite schools of opinion grew up, one which we may speak of as the "cellular" school of anaphylaxis, the other the "humoral."

In order to avoid further confusion, we may anticipate the analysis of these various opinions at once by stating that, as we now understand protein anaphylaxis, there can be little doubt about the fact that the most important phases of true protein anaphylaxis are the results of a reaction which takes place between the antigen and an antibody which is in some manner attached to, or in relation with body cells. To how great an extent an intravascular antigen-antibody reaction may play a minor rôle, we will discuss below; but, as to the primary importance of the cellular reaction in determining the anaphylactic phenomenon, there is little question.

The Cellular Site of the Anaphylactic Reaction.—The reasons why it is generally believed that such a peremptory statement concerning the cellular site of the anaphylactic reaction can be made, are the following:

In the first place, as we have stated above, particular attention was called to the possibility of a cellular site for the anaphylactic reaction by the interval which was found to be absolutely necessary between the injection of an antiserum and the development of the hypersusceptible condition in the injected animal. Both Friedemann and Otto found that when the serum, containing antibodies, was injected subcutaneously, the best results were obtained by administration of the antigen 24 to 48 hours after this. On intraperitoneal injection of the sensitizing serum, Doerr and Russ [15] obtained the best results by permitting an interval of 24 hours to elapse, and these investigators attempted to determine the minimum period necessary when fairly strong antisera were used, but could not shorten the interval successfully to less than 4 hours, even when the serum was injected intravenously. There were, indeed, certain exceptions to this observation in the earlier literature, such, for instance, as those published in 1910 by Biedl and Kraus [16] who claimed that they obtained immediate and severe symptoms in guinea pigs when they injected, intravenously, mixtures of horse serum, together with the serum of sensitized animals. Gurd [17] also, more recently, claimed he obtained reactions in guinea pigs upon the intravenous injection of anti-sheep rabbit serum, followed immediately by sheep serum. Exceptions to the observation of an interval in rabbits, we will take

[15] Doerr and Russ. *Zeitschr. f. Immunitäts.*, Vol. 3, 1909, p. 181.
[16] Biedl and Kraus. *Zeitschr. f. Immunitäts.*, Vol. 4, 1910.
[17] Gurd. *Jour. Med. Res.*, Vol. 31, 1914, p. 205.

up a little later in connection with a discussion of the humoral views, but all observations of this type are probably of minor significance and perhaps dependent upon a mechanism quite distinct from that of true anaphylaxis, relations which we will discuss under "anaphylatoxins." Except for these isolated instances, the literature is consistent in regard to the necessary interval and the fact is beyond question. Complementary to the observation of the interval is the determination by Doerr and others that, as the sensitive state develops, the antibody disappears from the circulation, and Doerr and Pick determined that anaphylactic shock could be obtained at a period when the circulation no longer contained any antibody. Moreover, earlier observations on active anaphylaxis by many investigators, showed that complete absence of demonstrable antibody in the circulating blood was not at all inconsistent with the existence of the sensitive state.

Another important plan of experimentation which had much influence in convincing investigators of the correctness of the cellular theory, were transfusion experiments. Manwaring [18] was one of the first to approach this problem in this way, by bleeding a sensitized dog extensively, and reinfusing him with the blood of a normal dog until there had been an almost complete replacement of blood. He found that the sensitized animal, in spite of the replacement of his blood, remained sensitive. Pearce and Eisenbrey [19] working with two normal and one sensitized dog, transfused the blood of one of the normal animals into the sensitized one, transferring the blood of the latter to the normal dog. "At the proper moment the normal dog containing the blood of the sensitized animal and the latter containing the blood of the normal dog, each received intravenously the toxic dose of horse serum." The normal dog having the sensitized blood did not react; the sensitized dog having the normal blood showed typical fall of blood pressure. Pearce and Eisenbrey drew the conclusion "that the phenomenon of anaphylaxis is due to a reaction in the fixed cells and not either primarily or secondarily in the blood." Similar experiments have since been done by Weil.

Coca [20] further fortified the cellular point of view by a similar method which is more than mere confirmation of the dog experiments of Pearce and Eisenbrey because he employed guinea pigs. He succeeded in perfusing actively and passively sensitized guinea pigs with the defibrinated blood of normal guinea pigs, until the original blood of the sensitized animals was reduced to a slight residue. Animals so treated could be kept alive for as long as 6 hours after the transfusion, in spite of the blood substitution, and remained sensitive. Again of considerable importance, and complementary to

[18] Manwaring. *Zeitschr. f. Immunitäts.*, Vol. 8, 1910, p. 1.
[19] Pearce and Eisenbrey. *Cong. Am. Phys. and Surg.*, Vol. 8, 1910.
[20] Coca. *Zeit. f. Immunitäts.*, Vol. 20, 1914.

the transfusion experiments, is the work of Weil [21] on the influence of large amounts of circulating antibody upon anaphylactic shock. Weil found that guinea pigs that had been either actively or passively sensitized, could be protected against anaphylactic shock by the introduction into their blood of large amounts of immune body just previous to the shock injection. It is true that Weil obtained no results when he used moderate amounts of immune serum only, and large amounts had to be introduced in order to give him this protection by circulating antibodies. He explained this by an avidity of the antigen greater for the sessile than for the circulating antibodies. Our own explanation, in keeping with the work of the writer with Young reported in an earlier chapter, would be that the phenomenon is not a question of avidities or chemical affinities, but rather attributable to the fact that in the circulation there is a colloidal protective action which prevents the sudden union of antigen and antibody, thus constituting perhaps the fundamental automatic protection against intravascular anaphylactic shock. However this may be, the fact that a sufficiently large amount of antibody present in the circulation will protect a sensitized guinea pig against shock seems to us very strong evidence in favor of the cellular conception.

Although it would hardly seem that with all this evidence further proof of the cellular site of the reaction would be necessary, nevertheless further incontrovertible proof was brought by the development of the isolated tissue technique, first by Schultz and later by Dale.

In 1910 Schultz began to work with what is now spoken of as the physiological method. He determined that smooth muscle—freshly excised from various animals—will react with contraction when brought into contact with serum. When such muscle was taken from anaphylactic animals after being thoroughly washed free of blood, it would react more energetically and to smaller amounts of the homologous serum than would similar tissue from normal animals. There are many interesting by-products of Schultz's work, such as the differences between fresh arterial blood and blood serum in their abilities to stimulate contraction, but this and other points will not be discussed at present. The important and incontrovertible fact established by Schultz is the changed reaction-energy or, in truth, "allergie" of the smooth muscle of anaphylactic animals to the stimulus of the sensitizing antigen. Dale [22] confirmed and extended these observations of Schultz. He removed the uteri from guinea pigs after thoroughly perfusing them with Ringer's solution to remove all blood. He then suspended them in baths of Ringer's solution and by the customary physiological methods measured the contractions following the addition of various amounts of foreign protein in the form of—among other things—horse serum and beef

[21] Weil. *Jour. Med. Res.*, New Series, Vol. 22, 1913, p. 497.
[22] Dale. *Jour. Pharmacol. and Exper. Therap.*, 1913, iv.

serum. He found that the uterus of an animal sensitized to horse serum would react to this substance in dilutions of 1:2,000 or 1:10,000, while the organ taken from a normal guinea pig reached its limit of reactionability at dilutions often less than 1:20. A uterus that had reacted strongly was found to be subsequently desensitized. A normal uterus could not—strangely—be passively sensitized by immersion into a solution containing serum antibodies. Richard Weil [23] has fully confirmed the principles laid down by Schultz and Dale. He incidentally also answered an objection to the conclusions of Dale and Schultz (never indeed a very valid objection) brought forward by Larson and Bell,[24] namely, that the reaction of the muscle tissue of a sensitized animal might be in part due to the fact that the blood, i.e., the antibodies, had not been entirely washed out of the tissue spaces by perfusion. Weil performed the very simple and ingenious experiment of injecting a normal guinea pig with large amounts of immune serum (anti-horse serum) and, after a few minutes, killing the animal. He then suspended the uterus in Ringer's solution in the usual manner without washing it completely free of blood. Contact with the homologous antigen produced no response. We may accept as definitely established by these researches of Schultz, Dale, and Weil that the fixed cells of anaphylactic animals possess an increased reaction-ability toward the antigen which is in no sense secondary to processes involving the circulating antibodies. Moreover, the work of Weil seems to indicate that desensitization of a passively prepared guinea pig deprives the uterus of its power to respond and that the gradual spontaneous diminution of hypersusceptibility on the part of the guinea pig is accompanied by an entirely parallel loss of reaction-capacity on the part of the isolated uterus.

Weil [25] also carried out a very ingenious series of experiments in which he tried to show that both antigen and antibody could be present on the tissue cell at one and the same time. Again using the isolated uterus method of Dale, he partially desensitized a guinea pig in the following way. The guinea pig was first actively sensitized with normal rabbit serum. After the lapse of a proper interval, the animal was reinjected, but this time not with normal rabbit serum, but with the serum of a rabbit immunized with horse serum, in other words, with rabbit serum containing antihorse antibodies. Thus, the effect of the second injection would be, in the first place, to act as antigen as far as its nature as rabbit serum is concerned, and in this capacity would partially desensitize the guinea pig to the rabbit serum element; on the other hand, this

[23] Weil, R. *Jour. Med. Research*, 27, 1913; 30, 1914; *Proc. Soc. Exper Biol. and Med.*, 1914, xi, 86.

[24] Larson and Bell. *Jour. Inf. Dis.*, Vol. 24, 1919, p. 185.

[25] Weil. *Jour. Med. Res.*, New Series, Vol. 25, 1914, p. 299.

rabbit serum administered at the second injection, also represented horse serum antibodies by virtue of which it could passively sensitize the animal to horse serum. On the day following this second injection, Weil tested the uteri of the guinea pigs and found them partially sensitive to rabbit serum, but also sensitive to horse serum. From this he drew the conclusion that since rabbit serum antigen was at the same time a conveyor of antibodies, the passive sensitization of the guinea pig against horse serum indicated that rabbit serum antigen must be attached in some way to the uterine tissue cells. The only possible objection to this line of very ingenious reasoning on the part of Weil is the development of our knowledge on the production of apparently protein-free antibodies, more recently developed by Huntoon in connection with pneumococcus antibodies. It is still possible that the presence of horse serum antibodies in the cells by virtue of the passive sensitization, might not necessarily indicate the presence of rabbit serum antigen as such. Yet, there is as much to be said on Weil's side as on the other, and the experimental evidence is all on his side. The reader may have a little difficulty in understanding this experiment unless he draws himself a simple diagram of the conditions in reading it. In describing Weil's work in a book of this kind it is difficult for a contemporary to omit deploring the fact that his untimely death deprived American anaphylaxis investigations of one of its most earnest and brilliant workers.

There is, thus, an incontrovertible mass of evidence available which proves without question that the site of the reaction which carries in its train the symptoms which we speak of as anaphylaxis, is predominantly on the cells of the body. Whether or not the intravascular reaction of antigen and antibody, which unquestionably to a certain extent takes place, has any, if even a minor importance, we will discuss directly.

In discussing cellular anaphylaxis, the experimental analysis has necessitated the investigation of tissues like smooth muscle, in which a definitely noticeable reaction was produced, and we have seen that it is probably the smooth muscle tissue, in guinea pigs in the bronchioles, in rabbits in the pulmonary vessels, in dogs in the herpatic circulation, which lead to the severe injuries probably responsible for death. This does not, however, mean necessarily, that other tissues of the body are not either acutely or subacutely injured. Indeed, many facts would indicate that sensitization by attached antibodies and subsequent injury is not by any means limited to individual tissues. The sudden drop of body temperature, the peculiar intestinal symptoms (vomiting, defecation and serosanguinous exudation of the intestinal mucous membrane) noticed by Weichardt and Schittenhelm [26] in dogs dying of protracted

[26] Weichardt and Schittenhelm. *Deut. med. Woch.*, 1902.

anaphylactic shock, the increased secretion of many glands and the symptoms referable to the nervous system, may, of course, be to some extent entirely secondary to changes in other organs. But it is also possible, and we may even say likely, that such occurrences indicate a generalized anaphylactic effect which in the case of many organs, of course, cannot be subjected to experiment with the same definiteness with which we can observe contractile tissues like the smooth muscle.

Similar in effect are the observations which we may classify as *local anaphylaxis*. In the general historical review we have already mentioned the phenomenon of Arthus, where a subcutaneous injection in a sensitive rabbit gives rise to œdema and eventual local necrosis. This phenomenon, however, has not been observed either in guinea pigs or in dogs with any degree of regularity. Lewis,[27] indeed, has described such a phenomenon in guinea pigs, but extensive experiments in our own laboratory, as yet unfinished, have failed to give anything like regular results in attempts to produce the Arthus phenomenon in these animals. Coca's suggestion that perhaps the conditions in rabbits differ from those in other animals in regard to this phenomenon because of a more severe and general effect upon the capillary walls, is worth considering, although other possibilities cannot be excluded. Whether or not the many local manifestations apparent in man on, let us say, the injection of antitoxic horse sera, or the quite severe and progressively increasing local reactions observed by anyone who has given the Pasteur treatment for rabies, can be regarded as evidences of local anaphylaxis, is a matter that cannot as yet be definitely determined. We, ourselves, are inclined to believe that this is the case. Coca, on the other hand, excludes such phenomena from classification with anaphylaxis for reasons which we will have occasion to discuss under the heading of serum sickness.

Auer [28] more recently made some very interesting and significant observations on local anaphylaxis. Auer was carrying out tests for hypersusceptibility on dogs which had been treated with horse serum some years before, and employed heavy doses of horse serum for reinjection. At the site of operation in the inguinal region, he observed two days later an extensive, thick œdema. In explaining this he assumed that the local reaction was due to the fact that a foreign protein, horse serum, was circulating in the blood and a certain amount of this protein, therefore, passed into the tissues along the wound during the development of the ordinary injury following operation. This was probably further increased by oozing of blood serum and plasma into the wound. Since the dogs were sensitized, his idea was that the skin and adjoining tissues, being

[27] Lewis. *Jour. Exp. Med.*, Vol. 10, 1908, p. 1.
[28] Auer. *Jour. Exper. Med.*, Vol. 32, 1920, p. 427.

sensitive, would respond locally to the protein so applied. In following this up he carried out analogous experiments in rabbits as follows: He reinjected sensitized rabbits with the homologous antigen and applied xylol, which is a powerful skin irritant, to the ears. In such cases he often obtained severe inflammation with the formation of crusts, and, occasionally, gangrene of the entire tips of the ears. Xylol applied in the same dosage and in the same way, to the ears of normal rabbits injected once with horse serum, and of sensitized but not reinjected rabbits, caused only a minor inflammation with a little œdema which disappears completely in two or three days. His explanation is that the lesion on the ears of the sensitized and reinjected rabbits after xylol, was anaphylactic in nature as the result of a local autoinoculation of the ear with the circulating antigen. The local autoinoculation was brought about, he thought, by the irritant action of the xylol which caused an inflammation and œdema at the site of application. An anaphylactic reaction now occurred because inflamed tissues were more active metabolically than the normal tissues, and the inflamed cells, therefore, affected more by the antigen than the normal cells. He concludes that this process may theoretically occur in any tissue of a sensitized animal under analogous conditions. These experiments are of considerable interest in perhaps pointing out possible anaphylactic injury where some mechanical or other cause of inflammation coincides with the presence of antigen in a sensitized subject.

The Factor of Injury in Anaphylaxis.—However well we may believe we understand the mechanism of anaphylaxis, and the site of the reaction, it is still obscure what the factor of injury actually consists in. Two chief lines of thought have been followed in the explanation of this, one of them attributing the injury to mechanical factors due largely to colloidal changes brought about by the antigen-antibody union, the other regarding the process as an intoxication with a poison formed in consequence of the union.

To the former category belong several theories in which the formation of a precipitate in or upon the cells has been suggested. Similar, also, is the idea of Nolf [29] who assumes a colloidal change by which fibrin is formed and becomes in some way attached to cellular elements. Because of utter lack of experimental data, or even methods by which this subject can be approached, we can pay very little attention to the physical theories. Moreover, most views involving physical processes, such as that of Jobling and Petersen,[30] imply the secondary formation of a toxic substance. Most of the theories implying the formation of toxic products will be discussed in our consideration of "anaphylatoxins." We wish merely in this

[29] Nolf. *Arch. Internat. de Phys.*, Vol. 10, 1910, p. 37.
[30] Jobling and Petersen. *Jour. Exper. Med.*, Vol. 20, 1914, p. 37.

place to discuss briefly the attempts to chemically define the poison possibly involved. Stimulus for investigations of this kind was given largely by the investigations of Biedl and Kraus[31] on peptone shock. DeWaele[32] had called attention to the similarity of the symptoms following the injection of peptone, to those of anaphylactic shock. Biedl and Kraus studied the phenomenon very carefully in dogs, and found complete analogy between anaphylactic manifestations and peptone shock. We have already called attention to the fact that they, furthermore, maintained the partial desensitization of sensitized dogs by preliminary peptone injections. While these observations have been amply confirmed, the chemically undefined nature of Witte's peptone prevents any degree of accuracy in chemical reasoning from these facts. Of more definite importance is the observation of Dale that histamin, a protein cleavage product, gives rise to symptoms in guinea pigs almost identical with those observed in anaphylaxis. Dale and Laidlaw[33] in their extensive studies on this substance, have worked out an almost complete analogy between the two phenomena. Moreover, as Wells points out, histamin, which is a histidin derivative, is present in every known complete protein. While these considerations are of great interest and should be held in reserve for possible bearing on future investigations, there are many points about the assumption that histamin is the actually effective toxic product developed in the course of anaphylaxis which are unproven and unlikely. We do not believe that Dale, himself, meant to draw any definite conclusions in this regard, but merely recorded the interesting observed facts in an objective manner. The suddenness with which anaphylactic shock supervenes on the injection of adequate amounts of antigen, alone renders it extremely difficult to assume that such an extensive cleavage of either the antigen or the cell substance could take place with the speed observed in shock. Moreover, Doerr[34] points out that histamin can be obtained from cellular materials only by extensive acid hydrolysis, and he seems to us quite logical in questioning whether it is possible to assume such a rapid and destructive cleavage as the result of a simple antigen-antibody union upon a cell. Moreover, he also points out that the production of histamin in a cell is quite incompatible with subsequent life of the cell, and from Dale's uterus experiments it is quite plain that a desensitized uterus may retain its contractile properties for a long time, sometimes not only returning to normal, but being unusually irritable.

The various opinions upon the production of toxic substances as

[31] Biedl and Kraus. *Wien. klin. Woch.*, No. 11, 1909.

[32] De Waele. *Bull. de l'Acad. Royale de Med. de Brux*, 1907, cited from Doerr.

[33] Dale and Laidlaw. *Jour. Physiol.*, Vol. 52, 1919, p. 355.

[34] Doerr. "Review of Anaphylaxis," *Erg. d. Hyg. & Bakt.*, etc., Vol. 5, 1922, p. 226.

a consequence of antigen-antibody union are extensively discussed in connection with the humoral theories and anaphylatoxin formation.

The Humoral Theories and the Development of the Anaphylatoxin Ideas.—We have seen that the most important point of departure for the conception of the cellular site of the anaphylactic reaction was the observation of the necessity of an interval between the injection of an immune serum in passive sensitization, and the development of the anaphylactic condition. From the beginning, a certain number of exceptions to this rule were noticed. Weill-Hallé and Lamaire [35] described experiments in which it was found that guinea pigs would react with typical anaphylactic symptoms if injected simultaneously with the serum of sensitized rabbits and the antigen. According to them, successful results in such experiments depended entirely upon the time at which the rabbits sensitized with the horse serum were bled. Friedemann [36] at about the same time published experiments on anaphylaxis in rabbits in which he obtained apparently typical anaphylactic symptoms on the simultaneous intravenous injection of antigen and the serum of sensitive animals. According to Friedemann, this was the best way to elicit an anaphylactic reaction in rabbits for he claimed that if the sensitive serum was injected 24 hours before the antigen, the reaction became indistinct. He concluded from these experiments that the anaphylactic poison, whatever it might be, is, in rabbits at least, formed in the circulating blood. In the same year, namely, 1909, Richet [37] obtained similar results with crepitin with which he sensitized dogs, bled them during the hypersusceptible period, mixed the serum with a harmless dose of crepitin, and injected the mixture into a normal dog. Violent anaphylaxis-like symptoms resulted immediately. He speaks of his experiments as "reaction anaphylactique in vitro." His experiments cannot be interpreted as having very much bearing on the present question because he worked with a substance primarily toxic, and of doubtful antigenic nature. However, Biedl and Kraus [38] shortly afterwards obtained immediate and severe symptoms in guinea pigs on intravenous injection of mixtures of horse serum, together with the serum of sensitized guinea pigs. Similarly, Briot,[39] reported similar observations in young rabbits, into which he had injected mixtures of horse serum and antihorse serum. Gurd [40] carried out similar experiments in guinea pigs with sheep and antisheep serum on simultaneous injection. Scott [41] reported similar observations on rabbits, and certain observations reported by Man-

[35] Weill-Hallé and Lamaire. *Compt. r. d. Soc. Biol.*, Vol. 65, 1908, p. 141.
[36] Friedemann. *Zeitschr. f. Immunitäts.*, Vol. 2, 1909.
[37] Richet.
[38] Biedl and Kraus. *Zeitschr. f. Immunitäts.*, Vol. 4, 1910.
[39] Briot. *Compt. r. d. Soc. Biol.*, Vol. 68, 1910, p. 402.
[40] Gurd. *Jour. Med. Res.*, Vol. 31, 1914, p. 205.
[41] Scott. *Jour. Path. & Bacter.*, Vol. 15, 1910, p. 31.

waring [42] indicate that analogous observations can be made in dogs. To this not inconsiderable mass of evidence we may add observations of our own which, though showing considerable irregularity, pointed in the same direction as the work reported by Friedemann. Indeed, as regards our own experiments on this subject, we may say that while we occasionally obtained severe unmistakeable anaphylaxis-like symptoms in rabbits on simultaneous or immediately consecutive injection, we could never be sure of successfully duplicating the result. If we summarize all the evidence concerning the question of the interval from the available literature, we believe that the following statement would come pretty close to a just appraisal. In guinea pigs there seems to be no question whatever about the necessity of some sort of an interval, and observations to the contrary can probably be explained upon some peculiarity of the experimental conditions which are not clear; but the overwhelming mass of evidence points to the necessity of some interval, probably not less than 4 hours. In rabbits and dogs, when large amounts of the two reacting substances are injected, passive sensitization may occur almost immediately, or within 5, 10 or 15 minutes. But this occurrence is irregular and cannot be obtained, in our opinion, with anything but a considerable dosage. We, ourselves, believe that the irregularity and the necessity for large dosage can be explained by the facts brought out in our studies on the simultaneous presence of antigen and antibody in the same animal from which we concluded by analogy, that the circulating blood normally contains a substance, probably associated with the albumins of the plasma, which, by a mechanism of colloidal protection, prevents the rapid union of antigen and antibody in the blood. This we regard as an automatic protective device which prevents the probably harmful effects of such sudden unions, making them gradual and slow, but is broken down by the use of large amounts of both substances and perhaps in individual animals may be, for some reason or other, defective.

However this may be, these investigations, coming early in the development of anaphylactic studies, gave rise to the assumption in the minds of many workers, that anaphylaxis might be the result of a meeting of antigen and antibody in the circulation, and many investigations were initiated in attempts to explain the mechanism by which this occurred.

The Anaphylatoxin Conception.—The concept underlying most of the work now undertaken in attempts to prove a humoral mechanism was the following: When a specific antigen meets its antibody the reaction between them gives rise to a toxic product, and this causes the characteristic symptoms. A similar idea, it will be remembered, is found in the original endotoxin theory of Pfeiffer. According to this, the action of the specific lysin liberated from

[42] Manwaring. *Zeitschr. f. Immunitäts.*, Vol. 8, 1910.

bacteria a preformed poison, the endotoxin. In 1902 Weichhardt,[43] bearing this conception in mind, subjected syncytial protein of rabbit placenta to the action of specific antisera and obtained substances toxic for normal rabbits. This work was done long before the days of anaphylaxis studies, and the results were interpreted in keeping with Pfeiffer's theory. However, as Weichhardt himself now claims, it is not unlikely that he was dealing with a phenomenon analogous to the ones we are now discussing. A similar opinion of the production of toxic substances by specific cytolysis was expressed by Wolff-Eisner [44] in 1904.

The idea is really a morphological one in which the "endotoxin" is regarded as something present in the antigen which is set free by disintegration of the cell. In applying this to serum anaphylaxis Wolff-Eisner [45] preserved this morphological simile in that he spoke of the dissolved protein antigen (serum, etc.) as "nur scheinbar gelöst" and "dass es erst durch die Lysine wirklich resorbierbar wird."

Indeed the sudden liberation of endotoxins by immune sera had been regarded by Pfeiffer and others as the cause of the rapid death often ensuing in *immunized* guinea pigs when more than a definite maximum of cholera spirilla or other organisms was injected. In all these opinions the basic conception was that certain bacteria contained a characteristic preformed poison (endotoxin) upon the pharmacological properties of which the peculiar symptoms caused by each organism depended.

Probably the most important of the earlier investigations along these lines, at least in its direct bearing on anaphylaxis, was the work of Vaughan and Wheeler,[46] published in 1907. Their conception of anaphylaxis takes root in the earlier investigations of Vaughan [47] and his pupils upon the extraction of a poisonous group from the protein molecule.

Vaughan and Wheeler [48] believed that the sensitizing and the toxogenic properties of the anaphylactic antigens are in truth contained within the self-same protein molecule; but can be chemically separated from each other. They were able to split egg albumen and other proteins by treatment with absolute alcohol (containing 2 per cent. NaOH) into 2 fractions—a toxic alcohol-soluble and a non-toxic alcohol-insoluble one. The former fraction gave protein reactions, and they regarded it as a true protein—while Wells,[49]

[43] Weichhardt. *Deutsche med. Woch.*, 1902, p. 624.

[44] Wolff-Eisner. *Centralbl. f. Bakt.*, Vol. 37, 1904.

[45] Wolff-Eisner. "Handbuch der Serum Therapie," p. 24, Lehmanns, München, 1910.

[46] Vaughan and Wheeler. *Jour. Inf. Dis.*, Vol. 4, 1907.

[47] Vaughan. *Trans. Ass'n Am. Phys.*, Vol. 16, 1901; *Jour. A. M. A.*, Vol. 36, 1901; *Am. Med.*, 1901; *Jour. A. M. A.*, Vol. 43, 1904.

[48] V. C. Vaughan, Jr. *Jour. A. M A.*, Vol. 44, 1905, p. 1340; *Boston Med. and Surg. Jour.*, Vol. 155, 1906.

[49] Wells. *Jour. Inf. Dis.*, Vol. 5, 1908.

considering the hydrolytic nature of the cleavage resorted to, considers this fraction as possibly a soluble peptone or polypeptid (the positive protein reactions being possibly due to amino acids). The non-alcohol-soluble, non-toxic fraction also gives protein reactions. Injections into guinea pigs of the toxic fraction produced symptoms not unlike anaphylaxis—but did not sensitize against protein. The alcohol-soluble portion was non-toxic and sensitized against protein in doses of 0.001 to 0.005 gm. Based on these results, their views of mechanism of anaphylaxis were as follows:

At the first injection a slow lysis (cleavage) of the injected protein gradually liberates a fraction, corresponding to the alcohol-insoluble substance—and this by its antigenic action gives rise to the formation, in excess, of an enzyme (lysin), which on reinjection brings about the rapid cleavage of the injected protein—with an explosive liberation of the toxic fraction and consequent symptoms.[50]

Nicolle believes that the injection of a protein into an animal induces the production in the subject of antibodies. These are preëminently two—albuminolysins, which cause its cleavage, and albuminocoagulins or precipitins, which coagulate and prevent the action of the lysin. At the time at which an animal is hypersusceptible or anaphylactic there has been a production of albuminolysins which cause cleavage of the protein, with the rapid liberation of toxic substances; but the albuminocoagulins or precipitins have not yet adequately developed. In a refractory animal the neutralizing action of the albuminoprecipitins prevents the harm which the lytic action might otherwise accomplish. The relative amounts of these two antibodies present in the circulation of the animal at any particular time determine whether the animal is anaphylactic or refractory or immune. This theory assumes arbitrarily the protective nature of precipitation, an idea which has no foundation in experiment and, in fact, is rendered extremely unlikely by more recent developments of our knowledge of the precipitating antibodies.

Given, then, a reasonable hypothesis in which anaphylaxis is associated with the cleavage of protein by lysis, given, in other words an antigen-antibody conception, it is but natural that experimenters should ask themselves: *What is the relation of the alexin to this cleavage?* For in all known lytic reactions, of course, the union of antigen and antibody leads to the absorption of alexin, by means of which, then, the lysis has been assumed to take place. This problem suggested itself to a number of the earlier investigators who attempted to approach it by determining whether or not the sera of sensitive animals, added to antigen, would fix alexin. Gay and Southard, Sleeswijk,[51] and others obtained negative results, while Nicolle and Abt,[52] and Doerr and Russ[53] obtained positive results. As far as this particular method is concerned, therefore, no conclusions can be drawn. Sleeswijk, however, has approached the question in another way and examined whether or not there is a diminution of alexin in the blood of an animal immediately after anaphylactic

[50] For the sake of completeness it is well also to mention Nicolle's theory, which, though attractive, is not borne out by recent knowledge concerning the nature of precipitins. See *Ann. de l'Inst. Past.*, Vol. 22, 1908.

[51] Sleeswijk. *Zeitschr. f. Immunitätsforschung,* Vol. 2, 1909.

[52] Nicolle and Abt. *Ann. de l'Inst. Past.*, Vol. 22, 1908.

[53] Doerr and Russ. *Zeitschr. f. Immunitätsforschung,* Vol. 3, 1909.

shock. He found that this was indeed a regular occurrence, and his results have been confirmed by Friedberger and Hartoch [54] and a number of others.

It was shown by these workers that, both in active and passive anaphylaxis in rabbits and dogs, as well as in guinea pigs, there is a definite and considerable diminution of complement immediately after anaphylactic shock.

The question now arises: What is the significance of this diminution of alexin? Do the animals die because of a sudden loss of circulating, physiologically necessary alexin, or does the alexin take an *active* part in producing the conditions which cause death?

Either of these possibilities might follow from the mere fact of alexin diminution, but the former—the possibility that complement depletion is the cause of death—was ruled out by Friedberger and Hartoch.[55] They showed that, by supplying fresh complement to sensitive animals at the time of reinjection, shock cannot be prevented. They now proceeded to demonstrate the *active* participation of complement in the production of anaphylaxis. They did this in an ingenious way which depended on utilization of the fact observed by Nolf,[56] Hektoen and Ruediger,[57] and others that hypertonic salt solution (1.5 to 2 per cent.) will prevent the combination of complement with its sensitized cells. By slowly injecting into sensitized guinea pigs 0.3 cubic centimeter of concentrated NaCl solution just before the injection of antigen they were able to markedly diminish anaphylactic shock—saving animals from injections which invariably killed the controls. The force of this experiment we think has been largely eliminated by work of the writer with Dwyer and Lieb [58] in which it was shown that the effect of the salt is upon the smooth muscle which is rendered less irritable by the salt and therefore less susceptible to the antigen.

An ingenious attempt to demonstrate the important rôle played by complement in anaphylaxis was that of Loeffler. Loeffler,[59] using guinea pigs sensitized with horse serum, completely depleted their complement by injecting intraperitoneally considerable quantities of sensitized sheep corpuscles. Tested by injection of horse serum one hour later no anaphylaxis occurred, while controls regularly succumbed.[60]

It seemed thus possible the complement or alexin might play an important active part in the production of anaphylaxis, and the

[54] Friedberger and Hartoch. *Zeitschr. f. Immunitätsforschung,* Vol. 3, 1909.

[55] Friedberger and Hartoch. *Loc. cit.*

[56] Nolf. *Ann. de l'Inst. Past.,* 1900.

[57] Hektoen and Ruediger. *Jour. Inf. Dis.,* Vol. 1, 1904.

[58] Zinsser, Lieb and Dwyer. *Proc. Soc. for Exp. Biol. & Med.,* Vol. 12, May, 1915, p. 123.

[59] Loeffler. *Zeitschr. f. Immunitätsforschung,* 8, 1910.

[60] For additional evidence pointing in the same direction see also Uhlenhuth and Haendel, *Zeitschr. f. Immunitätsforschung,* Vol. 3, 1909.

next logical step was to attempt to produce the anaphylactic poison by the action of alexin upon an antigen-antibody complex *in vitro.* This was first done, with direct reference to anaphylaxis, by Ulrich Friedemann.[61] Friedemann chose as his antigen-antibody complex the sensitized red blood cell after he had demonstrated by preliminary experiment that the basic anaphylactic experiment could be carried out in rabbits with washed beef corpuscles. He found that if 3 c. c. of such corpuscles were injected into rabbits and the injection repeated after 3 weeks anaphylactic symptoms were regularly elicited. He then allowed alexin to act upon sensitized beef blood *in vitro,* interrupted the action by cooling at a time just preceding the occurrence of hemolysis (to exclude the supposed toxic action of hemoglobin), and injected the supernatant fluid of such mixtures into normal rabbits. The result was marked illness resembling anaphylaxis, and Friedemann thus had succeeded in producing the anaphylactic poison *in vitro* under conditions as nearly as possible similar to those occurring in the circulation of the anaphylactic rabbit. In the conclusions drawn from his experiments he expresses the opinion that the poisons were not preformed in the red blood cells, but were formed by the proteolysis exerted by "amboceptor" and complement. In this statement he sets down the basic conception of the production of anaphylactic poisons now generally held.

Friedemann, then, in attempts to apply the same methods to the study of serum anaphylaxis, attempted to produce similar poisons by the action of rabbit alexin upon the washed precipitates formed by mixtures of antigen and precipitating sera. In this he failed—probably because of his choice of rabbits as subjects for experiment. Where he had failed, however, Friedberger [62] succeeded by using guinea pigs. Doerr and Russ [63] had previously shown that feeble symptoms of shock could be produced by the injection of serum precipitates into normal guinea pigs. With this additional evidence in favor of his reasoning, Friedberger proceeded as follows:

One c. c. of a rabbit serum which precipitated sheep serum in a dilution of 1 to 10,000 was mixed with 30 c. c. of a 1 to 50 sheep serum dilution. This was kept one hour at 37.5° C. and over night in the ice-chest, when a heavy flocculent precipitate had formed. This precipitate was washed to remove all traces of serum, and to it were added 2 c. c. of fresh normal guinea pig serum—as complement. This was again allowed to stand for 12 hours and then the supernatant fluid was injected into a guinea pig intravenously. In most cases the pigs so treated showed marked symptoms soon after the injection and died within a few hours.

[61] Ulrich Friedemann. *Zeitschr. f. Immunitätsforschung,* Vol. 2, 1909.

[62] Friedberger. *Berl. klin. Woch.,* 32 and 42, 1910; also *Zeitschr. f. Immunitätsforschung,* Vol. 4, 1910.

[63] Doerr and Russ. *Zeitschr. f. Immunitätsforschung,* Vol. 3, p. 181, 1909.

Friedberger concludes, therefore, that anaphylactic shock is a true intoxication due to a poison produced from the products of a precipitin-precipitinogen reaction by the action of a complement; he speaks of the formed poison as *anaphylatoxin.* This hypothesis although very attractive does not entirely meet with the facts as they have been developed since Friedberger's first work. The premises on which it is based assume in the first place that the poison or "anaphylatoxin" is formed out of the matrix of the antigen; further, it is definitely assumed that in the production of the poison after the antigen and antibody have met, the complement or alexin plays an active part. Friedberger's hypothesis as stated by him, moreover, assumed that the entire process took place intravascularly. However, even his own early experiments aroused some misgivings concerning the matrix of the poisons produced, for he found that they could be obtained as well when boiled antigen was used as when the fresh, unheated substances were employed, and the poisons were easily obtained from such organisms as the tubercle bacillus, which is extremely insoluble and unamenable to serum influence. It was also doubted whether one could truly assume the participation of the specific antibody or sensitizer in the production of Friedberger's poisons, since it soon developed that from bacteria, at least, the poison could be produced when the organisms were directly exposed to the action of fresh guinea-pig serum without the presence of any immune serum.

Experiments which soon cast a definite doubt on the assumption that the poisons were produced by a decomposition of the antigen were reported by Keysser and Wassermann.[64] These workers substituted insoluble substances like barium sulphate and kaolin for the antigen; that is, the precipitates or bacteria used in Friedberger's experiments. They found that if kaolin were treated with horse serum and then exposed to the action of guinea-pig serum or complement, poisons were produced identical in every respect to those produced by Friedberger's method. The conclusions they drew were that the poisons were produced, not by action of the complement on the antigen, but by its action on the horse serum absorbed by the kaolin. In other words, they transferred the matrix of the poison from the antigen to constituents in the serum itself, possibly the sensitizer or amboceptor. Bordet [65] also was able to show that poisons similar to those of Friedberger could be produced by the action of fresh guinea-pig serum on agar and recently Bordet has further shown that this is the case even when the agar has been by special methods deprived entirely of its nitrogenous components and represents simply a complex of carbohydrates. Agar-guinea-pig serum

[64] Keysser and Wassermann. *Folia serol.,* 1911, vii; *Ztschr. f. Hyg. u. Infectionskrankh.,* 1911, lxviii.
[65] Bordet. *Compt. rend. Soc. de biol.,* 1913, lxxiv, p. 877.

mixtures of this kind show an increase in total nonprotein nitrogen which would prove that the proteolytic action of the guinea-pig serum must have been active against its own proteins.

An interesting further development of this work has recently appeared in the experiments of Jobling and Petersen.[66] They showed that when bacteria are mixed with fresh active serum they adsorb the antienzymes normally present in blood. They have shown this experimentally and have proved that similar antienzyme removal can be accomplished by the addition of kaolin or agar, and by treatment with chloroform. Serums so treated become toxic, the actions of the poisons formed showing great similarity to that produced by Friedberger's anaphylatoxins. According to them, the poisons are formed because of the fact that antienzymes are adsorbed by the antigen, thus setting the normal ferments in the fresh serum free to act on their own serum protein.

It should be recalled that Friedemann, who was really the first one to show that the toxic substances could result from the interaction of fresh serum and sensitized antigens, although he succeeded only in doing this with red blood cells, suggested rather early that the success of such an experiment does not necessarily mean that the antigen furnishes the matrix entirely. He had studied the metabolism in anaphylactic poisoning and with Isaac has shown that the nitrogen output following reinjection in a sensitized animal is far in excess of that which could be derived solely from the injected antigen, and in this he has been confirmed by many other workers, notably by Vaughan. Moreover, recent investigations of Jobling and others have seemed to show that proteolysis is not necessarily a function of antibodies but is accomplished by non-specific protease in the blood.

Among the most elaborate studies of anaphylatoxin formation are those of Novy and de Kruif.[67] These workers produced toxic substances of this nature by incubating guinea pig serum with trypanosomes and with agar, and found that with this serum, as well as with rabbit and rat serum, poison production took place with great speed. In working with agar they found that the physical condition of the agar had important influence upon the speed of the reaction. When the agar was very finely divided in the gel state, toxic products were often obtained in less than five minutes. The amount of agar necessary was so slight that 0.0025 mg. of dried agar was enough to toxify a cubic centimeter of serum. This confirmed many of the observations cited above which completely excluded the production of the toxic substance from the substrate. In other words, these investigations, as well as other similar ones, showed that in all likelihood substances, such as bacteria, agar, etc., added to sera, acted indirectly in bringing about processes in the serum rather than in furnishing a material upon which the serum enzymes could act. The observations of Novy and de Kruif were extended to the well-known phenomenon first, we believe, noted by Doerr and Moldovan, that in the course of interrupted coagulation, blood may become primarily toxic. Blood apparently becomes toxic in the stages just preceding coagulation. According to Novy and de Kruif when the blood is drawn, catalytic substances form which change the fibrinogen into fibrin and produce anaphylatoxin-like substances in the process. These observations are of considerable importance in invalidating such experiments as those of Friedberger, Thiele and Embleton[68] and others, who tried to prove the existence of toxic substances in the circulation of animals, in various connections, by producing

[66] Jobling and Petersen. *Jour. Exp. Med.*, 1914, xix, No. 5.

[67] Novy and de Kruif. *Jour. Inf. Dis.*, 1917, 20, p. 499.

[68] Thiele and Embleton. *Zeitschr. f. Immunitäts.*, 19, 1913, pp. 643 and 666.

toxic symptoms when they injected fresh blood from one animal into another. Unless this is done with great speed, poisons may result even when the blood of normal animals is injected. According to Novy and de Kruif (we quote from their summary in the American Medical Association Journal for May 26th, 1917), the two reactions, both coagulation and toxification, go on hand in hand with the production of insoluble fibrin on the one hand and soluble anaphylatoxin on the other.[69] The disturbances which can bring about such transformations may be readily produced by the addition of almost any alien substance to sera, such as bacteria, trypanosomes, pepton, agar, starch, kaolin, etc., and identical reactions may be produced in vivo by the injection of these totally unlike substances into the circulation of an animal. Novy and de Kruif explain anaphylaxis by the fact that such a disturbance occurs when antigen and antibody meet in the body.

Along similar lines of reasoning are studies made upon the Abderhalden reaction by Bronfenbrenner[70] and others who found that in this reaction when the fresh serum of pregnant or immunized animals is brought together with the corresponding boiled protein, poisons appear, but that these poisons originate from the serum as a result of auto-digestion, and not from the substratum.

As a result of all the foregoing studies on the so-called anaphylatoxic substances we are forced to the conclusion that a great many processes associated with the contact of blood plasms with foreign materials may give rise to toxic products. This occurs easily in vitro, and may probably occur in vivo as well, and the poisons thus produced give rise to symptoms not unlike those of anaphylaxis. Moreover, the production of such substances by contact of fresh serum with specific precipitates in the hands of Friedberger, would render it not unlikely that the meeting of an antigen and antibody in the circulation could bring about such poison formation; and the experiments of Novy and de Kruif seem to indicate that the minutest amount of antigen and antibody union might bring about the result. Therefore, we must conclude that anaphylatoxin investigations, in themselves, are of the greatest importance in general pathological physiology, and that many phenomena in the body may perhaps be related to such poison formation. We would not, therefore, deny for one moment, the scientific importance of anaphylatoxin observations from which we may perhaps expect not a little sound scientific progress.

On the other hand, there is much which opposes the explanation of anaphylactic phenomena on the basis of anaphylatoxin formation. First and foremost among such objections is the fact of the cellular site of the reaction discussed in the preceding section. All workers who have inclined to an anaphylatoxin theory, have necessarily assumed the site of the reaction to be in the circulation. There is still a possibility, of course, that the union of antigen and antibody in or upon the cell many produce substances analogous to anaphylatoxins, either from the action of the plasma in close contact with the cells

[69] Conf. with Theory of Nolf.
[70] Bronfenbrenner. *Jour. Exper. Med.*, 21, 1915, p. 480.

or in the cell protoplasma, itself, but this is so purely speculative at the present time that it cannot alter the probability that true protein anaphylaxis cannot in any definite way, at the present time, be correlated with the formation of anaphylatoxins.

The extensive work which has been done on the formation of anaphylatoxin from bacteria, and the theories regarding the relationship of the poisons so produced to the explanation of the phenomena of infectious diseases will be dealt with in the chapter on hypersusceptibility in bacterial infection.

CHAPTER XVIII

SERUM SICKNESS, FOOD IDIOSYNCRASY, HAY FEVER, DRUG IDIOSYNCRASY

Serum Sickness.—We have mentioned that Rosenau and Anderson attacked the problem of hypersusceptibility primarily in the hope of casting light upon the nature and cause of the distressing symptoms which in human beings often ensue upon the injection of diphtheria antitoxin. It has been one of the staple objections of lay opponents to the use of antitoxins that the injections are apt to cause severe illness and occasionally death, and indeed a few cases are on record in which sudden death has followed the first injection of diphtheria antitoxin. Since it was known by accumulated clinical experience as well as by experiments like those of Bertin,[1] of Johannesen,[2] and others that the harmful effects were not dependent upon the antitoxin contents, but could be produced by injections of normal horse serum, it was but natural to bring these ill effects into analogy with the phenomena of hypersusceptibility. A large number of references to such antitoxin illness or SERUM SICKNESS have appeared in the literature since the first beginnings of the therapeutic use of sera, yet no careful analysis of the condition was made until von Pirquet and Schick,[3] in 1905, published their studies.

As a rule the results of serum injection have been mild and without danger, though sufficiently frequent and troublesome to call for thorough study and attempts to discover the prophylactic measures. As stated above, a few cases are on record in which sudden death has followed a single first injection. There are no reports in the literature known to us, however, of fatalities after second injections, although not infrequently such cases have taken on alarmingly serious aspects.

The percentage of incidence and the variety of symptoms have been the subjects of many reports. The most frequent and striking single occurrence has been an urticarial rash. Rolleston,[4] in a large series of cases, found urticaria in all but 17 of 289 cases of serum eruptions occurring between the first and tenth days after injection, and in all but ten of ninety-four later eruptions.

[1] Bertin. *Gaz. Méd. de Nantes*, 1895. Quoted from Levaditi.

[2] Johannesen. *Deutsche med. Woch.*, No. 51, 1895.

[3] Von Pirquet u. Schick. "Die Serum Krankheit," Deuticke, Leipzig, 1905. Also *Münch. med. Woch.*, 53, p. 67, 1906.

[4] Rolleston. *The Practitioner*, Vol. 74, 1905.

Rashes occurred in from 69.4 to 81.9 per cent. of the 600 antitoxin cases which Rolleston reports.

Joint pains commonly accompany the appearance of the rash, and these joint pains may offer considerable diagnostic difficulties. They are not often severe in actual discomfort, but may lead to a stiffness and difficulty in motion in cases in which tetanus antitoxin has been administered, giving rise to a considerable amount of worry. The writer has seen two cases in which the joints of the jaws, of the back of the neck and of the legs were so affected that slight rigidity and in one instance the difficulty of easily opening the mouth caused a good deal of uncertainty as to whether serum sickness or early tetanus were involved.

Albuminuria is occasionally present, and with it oliguria. Fever may accompany the appearance of the rash, but is rarely very severe. Œdema around the point of injection supervening upon a wheal, was noticed not uncommonly by Rolleston in cases of second injection of horse serum, and in such cases he occasionally saw vomiting, rigours and collapse within a few hours after injection, these severe symptoms being more apt to follow upon large than upon small doses. The lymph nodes in the area of injection may be enlarged and tender, and even glands not directly connected with the area of injection may enlarge. This symptom is quite regular and early.

While we have not desired to spend much time on the clinical description of this condition, we have sufficiently characterized it to show that there is a very real and definite sequence of events which may occur in human beings upon the injection of horse serum or other antisera.[5]

Von Pirquet and Schick have studied the condition with careful reference to a comparison between the symptoms occurring in subjects after a first injection of serum and those following upon repeated treatments. Their studies revealed the very important fact that the ill effects following a second injection were not only more severe than those occurring after the first injection, but developed after much shorter periods of incubation. In the ordinary "first injection" case the symptoms appear usually in from one to twelve days. After a second injection this incubation period may be considerably shortened and symptoms may appear in from five to seven days, the local and general reactions being much more marked than those subsequent to a first injection. Indeed, in some of the cases reported they may attain very alarming degrees of severity. This is the so-called accelerated ("beschleunigte") reaction of von Pirquet and Schick, and is different from the "first injection" symptoms only

[5] The statement made by Kraus, from observations in Argentine that bovine serum does not produce serum sickness has been contradicted by European workers. Doerr suggests the possibility of different dietetic habits to explain the discrepancy. Ref. See Doerr. "Ergebnisse der Imm., etc.," Vol. 5, 1922, p. 96.

in its greater severity and speedier onset. In addition to this, however, the "second injection" cases may show a train of immediate symptoms [6] (sofortige Reaktion), which occur within twenty-four hours after injection, and are characterized by marked local erythema and edema with often urticaria and constitutional disturbance. Both reactions may occur in the same individual, the "accelerated" reaction setting in as the "immediate" reaction subsides.

Again, one reaction or the other may occur alone. The analogy between the immediate reaction and the anaphylaxis of animal experiment is obvious. The cases may be classified on the basis of these reactions, according to von Pirquet and Schick, the nature of the reaction being, within certain limits, determined by the interval ensuing between the first and the second injection. Thus, when the interval was twenty-one days or less the immediate reaction alone was noticed. When the interval was between two and six months both the immediate and accelerated reactions were present, and when the interval was still longer (seven months or more) the accelerated reaction alone was present. Isolated exceptions to this are noted in the series of sixty-one cases so reported.

Currie,[7] who has made similar studies, confirms the results of von Pirquet and Schick in all essentials, and agrees with their statement that the nature of the reaction is chiefly dependent upon the interval between injections.

Percentage analyses of the various occurrences of serum sickness have been made by a number of writers, and can be found presented in compilations of the subject, like those of Doerr and of Coca referred to many times above. Longcope,[8] too, has analyzed this information with some thoroughness in his Harvey Lecture. Apparently, there are a number of different factors that determine the severity of serum sickness. According to studies of Weaver [9] culled from the literature of 801 reported cases, only about 10 per cent. developed the disease when quantities lower than 10 cubic centimeters were used, and in these cases the condition was usually a mild one. When 90 cubic centimeters, or over, were used, the disease was more severe, and over 75 per cent. of the cases developed it. Variations depending upon the source of the serum from different individual horses are too vague to be discussed, although a number of observers have reported such differences. The effect of the second injection varies, as we would expect from analogy with anaphylaxis, according to the interval elapsing between the first and second administration.

[6] Rankin in the *Lancet,* Dec., 1911, reports a case of "immediate" reaction 15 minutes after injection.

[7] Currie. *Jour. Hyg.,* Vol. 7, 1907. See also Goodall, *Jour. Hyg.,* Vol. 7, 1907.

[8] Longcope. Harvey Lectures, Ser. 11, 1915-1916, *Jour. Med. Sc.,* 152, 1916, p. 625.

[9] Weaver. *Arch. f. Int. Med.,* Vol. 3, 1909, p. 485.

Just as in guinea pigs, if one reinjects within a short period after the first administration, say a week or less, a refractory condition is produced, according to Longcope; and the development of sensitiveness is considerably prolonged. According to Goodall [10] however, eventually sensitiveness supervenes and is then quite severe. It is well to keep these facts in mind for purposes of future discussion, since they constitute analogies to the condition of true protein anaphylaxis in guinea pigs and other animals. If the second injection is made two weeks or later, after the first, the patients are likely to have the local immediate reaction which has been mentioned above, within less than an hour after the injection, and the general reactions may come on within less than a day, and then be accompanied, on occasion, by severe respiratory difficulties. In such cases the "Beschleunigte" reaction of von Pirquet and Schick is often noticed a few days after the injection. It is well again to note here the analogy between the immediate local reaction and the Arthus phenomenon observed in rabbits.

The period after the first injection at which the *immediate* reactions are most likely to occur are stated by Longcope from his study of the literature and from his own observations to lie between the 35th and 80th days after the primary inoculation, in which instance it occurs in 60 per cent. of the cases, if enough serum is injected. The accelerated or "Beschleunigte" reaction occurs in injections made three months after the first injection. From a practical point of view, it is important to consider just how serious the danger of death may be in such cases. Specific statistical studies have been made on this question by Park,[11] and by Cuno,[12] according to whom subcutaneous injection never causes death, though the symptoms may be extremely severe. But isolated cases of death have occurred after intravenous use. One such case was reported by Koch [13] in 1915. In view of the frequency with which serum is injected intraspinally in the treatment of meningitis and some other conditions at the present time, Longcope's summary of results of this procedure is worth quoting. According to Auer who summarized these cases in Forscheimer's System, and whom we quote from Longcope, death after second injection into the spinal canal has been reported by at least three observers.[14] Auer regards these as results of a local immediate reaction in the meninges, analogous to the Arthus phenomenon.

The analogy between serum sickness and anaphylaxis, is, there-

[10] Goodall. *Jour. Hyg.*, 7, 1907, p. 607.

[11] Park. *Trans. Ass. Amer. Phys.*, 28, 1913, p. 95.

[12] Cuno. *Deut. med. Woch.*, 40, 1914, p. 1017.

[13] Koch. *Wien. klin. Woch.*, 52, 1915, p. 685.

[14] Hutinel. *Presse Med.*, 18, 1910, p. 497. Grysez and Dupuich, cited from Longcope, *loc. cit.* Archard and Flandin. *Compt. rend. d. l. Soc. Biol.*, 73, 1912, p. 419.

fore, a pretty close one. It was early shown that the antitoxin contents of the sera had nothing to do with the occurrence of the serum sickness, but that this depended upon the foreign proteins rather than upon its antibody contents. Moreover, the shortening of the incubation time upon second injection and the greater severity of the symptoms following second and third injections, makes analogy with the protein sensitization of guinea pigs a very persuasive one. If serum sickness is truly an anaphylactic phenomenon, however, it is still by no means clear why symptoms should at all ensue after the first injection. The views of von Pirquet and Schick upon this were based upon the fact that in many cases the incubation period of serum sickness after first injection is a long one, namely, 8 to 12 days. Their idea was that, as the antigen is gradually brought into contact with the cells of the body, antibody begins to form, but that antibody formation precedes considerably the removal of all the antigen from the body. That this was a possibility was indicated by the studies we have many times referred to of von Dungern and others, that antigen and antibodies may be present in the circulation of an animal at one and the same time, especially after the injections of considerable quantities. The newly formed antibodies might, then, react with the antigen residue still present. In supporting this idea, they investigated the formation of precipitins in the blood of a rabbit injected with horse serum, finding that precipitins occurred as early as the eighth or ninth day. Wells [15] investigated this condition in human beings in which he found that the incubation of precipitin production varied, but was not dependent particularly upon the amount of antigen injected; nor was there any regularity in the incubation period of the serum sickness, which was very variable and sometimes not longer than four days. The matter has been extensively studied of recent years by Longcope and Rackemann [16] and by Longcope, Mackenzie and Leak.[17] Longcope and Rackemann concluded from their experiments that the blood serum of most patients who suffer from an attack of serum disease following injection of horse serum, show precipitin for horse serum, and that such serum could passively transfer the anaphylactic state to guinea pigs, thus bearing out the work of Hamburger and Moro,[18] and of Wells. They found, also, that these antibodies could not be demonstrated in the blood serum of patients treated with horse serum who did not later present symptoms of serum sickness, and that the appearance of the antibodies shortly preceded recovery from the disease. The continuation of this work by Longcope and his collaborators on 19 further cases led to the conclusion that there is a definite relationship be-

[15] Wells. *Jour. Inf. Dis.*, 16, 1915, p. 63.
[16] Longcope and Rackemann. *Jour. Exp. Med.*, 27, 1918, p. 341.
[17] Longcope, Mackenzie and Leak.
[18] Hamburger and Moro. *Wien. klin. Woch.*, 16, 1903, p. 445.

tween antibody formation and the severity of the symptoms and that severe reactions come chiefly and "perhaps exclusively" in those individuals who develop a high concentration of precipitin in the circulation. They found that in such patients the horse serum is apt to disappear from the circulation soon after a large amount of precipitin appears. They differentiate between individual patients by stating that some individuals seem to be relatively insusceptible to serum reaction, and that these are the cases in which little or no precipitin is formed. The tables given by the last named group of observers show a number of cases in which a failure of the development of serum disease was accompanied by a failure of antibody production, and also a considerable number of cases in which a number of symptoms attributable to serum disease preceded the observation of any antibodies in the blood. Indeed, in one case symptoms seemed to appear 24 hours after the injection, in several cases after 2 days, and in a number of cases between the fourth and seventh day, which would be in keeping with Wells' observation of a frequent incubation time as short as 4 days, the height of the serum disease usually, however, coming on later than this.

We can, thus, state without going into extensive tabulation of statistics that in analogy with anaphylaxis, serum disease comes on after the injection of an antigenic substance; that when it comes on after first injection the incubation period may be, but is rarely less than 5 days, and is usually longer than this; that, as von Pirquet, Goodall,[19] and Currie [20] have shown, second and subsequent injections usually give rise to a shortening of the incubation period; and there is at least considerable reason to believe that antibody formation and serum disease are in some way related.

As against these observations, Coca has entered objections, separating serum disease definitely from true anaphylaxis, and classifying it with idiosyncrasies, such as drug allergies. One of his arguments is the clinical parallelism between the symptoms of serum disease and drug allergies in regard to fever, skin rashes, the polymorphous form of these rashes, œdema, joint pains, leukopenia, etc. As far as this clinical argument is concerned, we are inclined to give it very little weight. As Doerr points out, there are at least as many similarities between human serum disease and anaphylaxis in animals as there are similarities between drug idiosyncrasies and human serum disease. He cites such occurrences as the secretions of tears, increased secretion of the nasal and gastrointestinal mucous membranes, and difficulties of respiration; and as far as the eruptions are concerned, he calls attention to the apparent discomfort of the skin and the scratching of guinea pigs in non-fatal anaphylactic shock. To this we may add that, after all, the symptoms of true anaphylaxis

[19] Goodall. *Jour. Hyg.*, 7, 1907, p. 607.
[20] Currie. *Jour. Hyg.*, 7, 1907, p. 35.

are so various in the manifestations as observed in different species of animals in which we can experimentally determine the anaphylactic mechanism, that it would be surprising if man reacted to the anaphylactic condition with symptoms entirely similar to those prevailing in lower animals.

Coca criticizes the attempts to show parallelism between antibody reaction and the onset of serum disease by stating, in the first place, that the symptoms generally preceded the appearance of precipitin and sometimes disappeared while precipitin and horse serum were still present together in the patient's blood, and in the second place because the symptoms sometimes continued after precipitin was no longer demonstrable, and occasionally occurred in patients in whom no precipitin production whatever could be shown. These are interesting suggestions, but do not seem to us convincing. In the first place, we must assume that antibodies are formed in or on the cells of the body, and that these cells may, therefore, be sensitive a considerable time before demonstrable precipitins occur in the circulation; and, indeed, our own point of view about antibodies is such that we would expect a not inconsiderable amount of antibody actually to occur in the blood before a visible precipitin reaction could be elicited. Thus, our own view in which the identity of precipitins with other protein antibodies is a factor, would incline us to expect, as found by Longcope and his collaborators, that sensitiveness of the cellular complexes of the body would precede successful demonstration of precipitins in the blood. That symptoms may continue after precipitins have disappeared, and that symptoms could occur in individuals in whom no precipitins could be demonstrated, does not by any means signify that in such individuals there could not have been antibodies sessile on the cells capable in this site of reacting with the antigen. Indeed, the very arguments Coca uses in this case would seem to indicate that in this instance he favored an intravascular antigen-antibody reaction, in spite of the fact that his own work has been of considerable contributory value in locating the anaphylactic reaction upon the cells in lower animals.

A type of reaction which Coca mentions in his argument, which cannot be explained by any such reasoning as that which we have employed above, is that in which symptoms appear almost immediately upon first injection. This, as he rightly remarks, seems to offer serious obstacles to the harmonizing of serum disease with anaphylaxis. However, these cases are not very frequent, and while we do not wish to imply that we possess a positive explanation for this phenomenon, nevertheless, we believe it worth considering that it is not at all impossible that certain individuals may be accidentally sensitized to various proteins, either through the intestine or through some other mucous membranes as suggested by Richet, and by Rosenau. Again it is important to remember that patients sys-

tematically sensitive to horse serum usually show immediate cutaneous reactions to this antigen (Longcope and Rackemann), and that the writer, with J. T. Parker, noted the development of similar skin sensitiveness in horse serum sensitized guinea pigs coincident with the development of the general anaphylactic state. Our own observations on monkeys, I think, have some bearing on this subject in that we found that it was extremely difficult to produce anaphylaxis in several of the lower monkeys, but that when any symptoms at all were observed (these symptoms in one case being strikingly similar to human serum disease), they were associated with some precipitin formation.

A line of reasoning in which it is not so easy to reply to Coca is the apparent difficulty of desensitizing human individuals. Doerr replies to this by stating that these difficulties must be interpreted with proper consideration of quantitative relations and the degrees of sensitiveness, factors which have not been sufficiently considered in desensitizing experiments on man. As a matter of fact, Doerr, who discusses Coca's objection in this respect at considerable length, cites a number of cases in which desensitization has been successfully reported by clinicians, both in conditions of horse serum sensitiveness, as well as in food idiosyncrasies. Schloss particularly has reported cases of this kind, and while the desensitization of human beings does not seem to be as simple as it is in animals, perhaps because we cannot treat as drastically and have no measure of the degree of sensitiveness, there is sufficient evidence available to at least make Coca's argument in this respect a questionable one.

We are, therefore, inclined to believe that for the present, the parallelism between anaphylaxis and serum disease in man is sufficiently striking to incline us to favor the fundamental identity of both processes, admitting, however, that man seems to be an animal in which sensitiveness to the anaphylactic experiment is fortunately considerably less than in other animals, for reasons perhaps associated with peculiarities of antibody formation, or with the relative distribution of antibodies between cells and circulation. It is our opinion that this relationship has not been sufficiently considered in reasoning about anaphylaxis; for instance, the equilibrium established between cells and circulation in regard to the relative amounts of cellular and circulating antibodies must be a matter dependent both upon the species of animal and upon the temporary conditions prevailing respectively in the circulation and in the cells. As far as the influence of animal species is concerned, we know that while guinea pigs are the most delicate animals to anaphylactic reactions, thus arguing for a powerful concentration of antibodies in or upon the cells, they are much less favorable for the production of circulating precipitins than are, for instance, rabbits.

Desensitization in Serum Sickness.—Although the danger of

fatal accident in the subcutaneous administration of antitoxic sera is not a very great one, yet the increasing frequency of intravenous administration in, for instance, the use of pneumococcus serum and the isolated reports of cases of fatal result, render it desirable not only to gauge the sensitiveness of the patient, but also to apply some of the principles of desensitization, determined for animals, in the procedure. In view of the apparently reasonable parallelism between skin reactions and general hypersensitiveness, it has become a custom before giving such cases an intravenous dose of the serum, to apply an intradermal skin test with about 0.01 to 0.02 c. c. of the horse serum diluted 1-10 with salt solution, that is, a total amount of 0.002 c. c. of the actual serum. A control with salt solution should be made to guard against errors from urticarial tendency in the patient. If a positive skin reaction, that is, the rapid development of an urticarial wheal over the injected spot appears which gradually fades in the course of one or two hours, great care in injecting should be used. Desensitization as advised by Cole and his collaborators can be attempted by starting injections of the patient with 0.025 c. c. of serum, and repeating this subcutaneously at one-half hour intervals, doubling the size of injection. If in this way 1 c. c. has been given without reaction, they continue with 0.1 c. c. at one-half hour intervals, intravenously. The process, of course, is based on the observations of Friedberger, Besredka and others, cited in a preceding section, and made upon guinea pigs, and can be gauged in speed and amounts only by careful observation of the patient. In addition to this safeguard, the procedure first applied by Friedberger should be used, namely, the dilution of the therapeutic serum to at least 50 per cent. of its original concentration with salt solution and the very slow intravenous injection by gravity, in such a way that the first 10 c. c. occupies at least ten minutes.

Differentiation between True Anaphylaxis and Idiosyncrasies.—Before going on to the consideration of other forms of hypersusceptibility, we insert this brief section for the purpose of making our opinions concerning the inter-relationship of the various forms of hypersusceptibility perfectly clear. In the preceding sections we have dealt with protein anaphylaxis and with serum sickness, a condition which we believe to be more closely allied to protein anaphylaxis than to any other form of hypersensitiveness. We do not think that there is sufficient ground for excluding serum sickness from the protein anaphylactic phenomenon, if all the conditions and analogies are properly taken into consideration. In the following sections, we shall take up conditions of hypersusceptibility to various food substances, the disease spoken of as hay fever, and the so-called drug idiosyncrasies. It is impossible to state any specific criteria by which we can sharply separate all of these conditions from true anaphylaxis. To base this partly on clinical symptoms as Coca has done we do

not believe is valid, nor can the entire class be separated from anaphylaxis on the basis of the claim that the inciting substances are non-antigenic, for, in the food idiosyncrasies and even in hay fever, in our own opinion, the possibility of the participation of a true antigenic substance is not only possible, but likely. We think the problem may be justly stated in the following way: In true protein anaphylaxis we have a well defined group of phenomena in which the inciting substance is always an antigen; in which it can be proved that the reaction depends upon the sudden union of antigen and antibody within the animal body; which can be easily and always elicited by artificial active sensitization; and can be passively transferred with the serum containing the antibodies. The site of the reaction can be proved to be predominantly cellular, and specific desensitization is easily possible. Of great importance, moreover, is the fact that true anaphylaxis, as far as we know, is not inheritable, except in so far as placental transmission of antibodies from mother to offspring may occur, thereby conferring a temporary passive sensitization; but true bioplastic inheritance in true anaphylaxis is not known.

In contrast to this, let us characterize the so-called idiosyncrasies: In these conditions the inciting substance may or may not be a recognizable antigen. In many of the food idiosyncrasies the antigenic nature of the inciting substance is unquestionable; in hay fever there is an antigen in the pollen, but we are not ready to state that this antigen is the responsible ingredient; in drug idiosyncrasies, the original inciting substance, as such, is non-antigenic. Most important, moreover, is the fact that for these so-called idiosyncratic conditions, a definite possibility of inheritance has been established, although again let us emphasize that inheritance is not a factor in all cases. These various differences have long been recognized by a considerable number of observers. Doerr has again and again called attention to many of them, and we, ourselves, have discussed some of them, particularly in relationship to bacterial hypersusceptibility, but they have been brought together most clearly of recent years by Coca, who bases much of his arguments upon the important studies of Cooke and Vander Veer.

We do not ourselves, however, believe that in these phenomena we can establish a sharp line of separation between protein anaphylaxis and the idiosyncrasies, but that the conditions shade into each other by many gradual transitions, dependent upon a number of factors, some of which will be discussed in our concluding section.

In this discussion we have omitted bacterial hypersensitiveness, a matter which can be most easily discussed in a separate section.

Food Idiosyncrasies.—Hypersensitiveness to the ingestion of various kinds of meat, of milk, of eggs, sea food, strawberries, etc., has again and again been reported. In this general category, also, we may include hay fever, and, with it, such conditions as horse asthma

and the hypersusceptibility observed in many individuals to the emanations from various animals with which they come in contact. In such patients even temporary and brief contact with the responsible animal may call forth reactions of extreme violence, clinically quite similar to the severe seizures of hay fever. The inclusion of horse asthma and allied conditions with food idiosyncrasies and hay fever, instead of with serum sickness, though in both cases an antigen derived from an animal is responsible, is based very largely upon the similarity of clinical pictures and upon the extremely minute dosage which in these conditions can call forth the most violent reactions. Also, as stated in the preceding section, these conditions, like food idiosyncrasies and hay fever, seem in many cases to be governed by inherited predispositions. We believe it quite well worth noting in addition to this, that food idiosyncrasies and such conditions as hay fever and horse asthma, have another common feature which may be more important than hitherto supposed, namely, they are all conditions in which the sensitization seems to take place by the path of a mucous membrane, the intestinal canal in the one group, the upper respiratory passages in the other. In the former the sensitization seems entirely an intestinal one, and the symptoms usually, if not always, present some form of gastro-intestinal seizure. In the latter, hay fever, horse asthma, and allied conditions, the sensitization is always, as far as we know, one involving the upper respiratory tract, and while systemic symptoms are to some extent noticed in the attacks, nevertheless, the disturbances of the mucous membranes of the nose, throat and bronchial passages predominate, as though a condition of local sensitization played an important rôle in the mechanism. It is the problems of local sensitization about which we know least in the analysis of hypersusceptibility.

The frequency of such forms of hypersensitiveness is much greater than formerly suspected, for there are probably cases of latent idiosyncrasy in which accidental freedom from the harmful contact has prevented recognition of the condition and which, therefore, have been revealed only by the experimental testing of human beings by the skin and conjunctival reactions.

The relative frequency of the various kinds of sensitization have been studied by a number of observers. Hay fever will be discussed in a separate section. In regard to other forms, however, it is interesting to note that Cooke and Vander Veer [22] in a study of 318 cases, found 37 sensitive to horse, 35 to strawberries, 28 to shellfish, and 27 to fish.

Schloss [23] has particularly studied the hypersensitiveness occurring in children to eggs, cows' milk and other forms of protein; and similar studies have been made by many other observers. In

[22] Cooke and Vander Veer. *Jour. Immunol.*, 1, 1916, p. 201.
[23] Schloss. *Amer. Jour. Dis. Child.*, 3, 1912, p. 341.

such individuals a variety of apparently specific reactions may occur. Schloss [24] analyzes his cases into a number of different clinical groups. The most severe are those in which the food is followed by so rapid and violent a reaction that the child does not swallow it. Immediate discomfort leads to spitting and vomiting within a few minutes, and this is followed by swelling of the lips, tongue and mouth, with which severe general symptoms develop, sometimes sufficiently serious to be termed collapse. During recovery from this, general eruptions of an urticarial type may develop. In other cases, again, conjunctival congestion and attacks of sneezing not unlike hay fever, may occur, and in the more severe ones of this type, typical attacks of bronchial asthma supervene. Urticarial eruptions may occur alone as the only evidence of the hypersusceptibility, or may accompany any other form of the disease. Agioneurotic œdema occasionally results, either with or without urticaria. It is of importance that urticaria is not the only form of skin eruption which is noticed, but Schloss has been able to trace various forms of erythema and even eczema to food intoxication. An important group studied by him, furthermore, is one in which the disturbances are chiefly gastro-intestinal, with vomiting and diarrhea, and the frequency with which these symptoms occur alone or together with other symptoms, is important in view of the remarks we have made above concerning local sensitization.

All this evidence gathered by Schloss, Bruck [25] and many others show that a considerable variety of clinical disturbances may be specifically related to hypersensitiveness to many different foodstuffs, and these studies have again brought out the important fact that general sensitiveness is associated with cutaneous hypersusceptibility which can be determined by the intracutaneous injection of minute amounts of the inciting substances.

Another fact of considerable importance is that a patient may be susceptible to a number of different foodstuffs at the same time. One of Schloss's cases was, at one and the same time, susceptible to eggs, almonds and oatmeal, and he and others have mentioned similar cases in which a multiplicity of proteins would produce symptoms in one patient. Cooke and Vander Veer's study of 551 cases, revealed 318 which were sensitive to one protein only, 172 which were sensitive to two, 42 which were sensitive to three varieties, and 19 to more than 3 inciting substances; 42.3 per cent. of their cases, therefore, were sensitive to more than one substance.

How may such sensitiveness be explained? From the careful studies of many clinicians it appears that two classes of patients occur. In a definite proportion of these there is reasonable evidence for complete analogy with the anaphylactic experiment, in that sensi-

[24] Schloss. *Amer. Jour. Dis. Child.*, 19, 1920, p. 433.
[25] Bruck. *Arch. f. Dermat. & Syph.*, 96, 1902, p. 241.

tization is developed a certain length of time after the first contact with the responsible foodstuff, the first ingestion causing no symptoms whatever. Such cases again raise the problem of the possibility of sensitization by way of the intestinal canal. Ordinarily, of course, we know that sensitization is not easily accomplished unless the antigenic agent is administered to the body by some path which avoids digestion in the intestine and under ordinary circumstances we know that proteins, as such, will not pass through the intestinal mucosa. However, Rosenau and Anderson [26] have shown that sensitization of this kind can be accomplished in guinea pigs, and Wells [27] has made similar observations. Schloss [28] accomplished the same thing by feeding guinea pigs for from 10 to 32 days with egg-white. He not only found that in some of his guinea pigs, animals so fed and subsequently tested by intraperitoneal injection with egg-white became definitely ill, but, in other experiments, also determined that intraperitoneally sensitized guinea pigs might show many of the symptoms of anaphylaxis if fed considerable amounts of egg-white 13 days after the sensitization, following a short period of starvation to empty the bowel. In these animals it is interesting to notice that one of the immediate symptoms was the development of watery stools. The experiments done by Schloss on the basis of a large experience and great experimental care, would seem to permit no question concerning the fact that guinea pigs can be sensitized by feeding, and that sensitized guinea pigs can be intoxicated by feeding. That similar conditions may occur in human beings seems more than likely, in view of the fact that the intestinal history of all human beings from the earliest age, will show a great many periods of abnormal intestinal function due to inflammatory conditions of various kinds, coincident or perhaps sometimes secondary to over-feeding in which proteins are copiously ingested. In regard to these cases, then, analogy with protein anaphylaxis can be taken to be a close one, and the burden of proof would rest definitely upon those who doubted such a classification.

On the other hand, there are the so-called *Congenital* cases in which there may be absolutely no traceable previous contact with the harmful foodstuff. This causes its symptoms upon first ingestion and, in many of these cases, a definite family history of hypersensitive conditions in the immediate ancestors of the patient can be traced. This class of so-called congenital cases is not at all infrequent. In 1912 Schloss reported an interesting case of a boy of eight, in whom urticaria and other symptoms followed the ingestion of egg, almonds and oatmeal. The oatmeal sensitiveness appeared some time after a preceding feeding with oatmeal, the first ingestion having caused

[26] Rosenau and Anderson. *U. S. Hyg. Lab. Bull.*, 29, 1906.
[27] Wells. *Jour. Inf. Dis.*, 8, 1911, p. 66.
[28] Schloss. *Loc. cit.*

no symptoms at all. In the case of the almonds and the egg, however, no relationship with previous contact could be determined. The child showed positive skin reactions to the three proteins, both in their native and purified forms, which was so intense that on some occasions positive reactions were obtained by mere contact of the protein with the unbroken skin. Schloss was able to transfer the egg sensitiveness, passively, to guinea pigs by injections of the patient's serum. It is of great interest to note that careful feeding of ovomucoid desensitized the patient not only to egg, but, to some extent, to the other two substances involved. This is of great interest in indicating the bare possibility that an antigenic group reaction may, to some extent, explain cases of this kind. This however may also be explained by experiments such as those of Dale on non-specific desensitization (see previous section on this subject).

Such an explanation, however, cannot be applied to a great many other cases. It can hardly be questioned any longer that true inheritance may play a rôle in food idiosyncrasies, hay fever, etc., and is an important characteristic of these conditions. The problem has been studied by a great many observers, but most extensively by Cooke and Vander Veer. Their work was carried out by a careful experimental plan with a clinical material so extensive that their conclusions deserve the most serious consideration. They studied 504 cases of demonstrable hypersensitiveness. In 260 of these there was no history of hypersensitiveness in the immediate ancestors. In 205 of them there was a positive history on the one side, and in 39 a positive history on both sides. Thus, 48.4 per cent. of the sensitive cases had a traceable history of inheritance, as against 14.5 per cent. of ancestral sensitiveness for normal individuals. This discrepancy in itself seems significant, and becomes more so if we consider the possibility, referred to above, of latent hypersusceptibilities which could not be revealed in any manner in the last few generations because of the relatively recent introduction of the skin and conjunctival tests.

According to them, the child is not born hypersusceptible, but develops this susceptibility some time after birth. In those with a double inheritance, the susceptibility developed, in a considerable proportion of the cases, within the first five years. With a single hereditary history, the most frequent age for the development of the condition was between ten and fifteen years, and in those with a negative history, twenty to twenty-five years was the most frequent age for the development of symptoms. From this they conclude that the inheritance is not that of a specific hypersensitiveness, but rather represents merely the transmission of a tendency to hypersusceptibility. This, too, is indicated by the fact that the specific sensitiveness of the child may be for a substance quite different from that afflicting the parent. This curious fact might incline one to argue

that such a supposed inherited tendency might mean nothing more or less than the variable reaction capacity for the production of antibodies noticeable in laboratory animals; for anyone who has ever extensively immunized rabbits or other animals with various antigens, has found very marked differences in antibody production in individual subjects, irrespective of physical condition, feeding, etc. However, Cooke and Vander Veer think they excluded this possibility by observing reactions to horse serum injections in antitoxin treated individuals, some of whom were naturally idiosyncratic to food or pollen, the others being normal. They found practically no difference between the two groups in regard to the development of skin tests to horse serum after antitoxin administration. Thus, it does not appear that idiosyncratic individuals are more easy to sensitize artificially than are normal ones.

Similar results were obtained by Schloss in a smaller series. Schloss summarizes his own studies in stating that he was able to obtain satisfactory family histories in 80 cases. In 40 there was a definite history of allergy in parents, brothers and sisters. In 7 other cases no history of allergy in the parents could be obtained, but it was positive for the grandparents. In the remaining 33 cases, no family history of allergy could be obtained, but of these 16 had developed symptoms on the first ingestion of the responsible food, though the history had been negative. In 17 of the 80 cases no family history was obtained, and the characteristic symptoms did not appear until the food had been once or several times partaken of, previous to the development of symptoms without causing ill effects. In this group, therefore, it seems reasonably sure that the sensitiveness was acquired. Schloss's figures give a relative estimate of the numerical relationship between the congenital and the acquired cases.

That the inheritance follows Mendelian principles in general is also apparent from the researches of Cooke and Vander Veer, though the difficulty of obtaining complete data and of finding families with sufficiently large numbers of children, hampered them considerably.

We have stated the arguments in favor of the acceptance of inheritance in a group of so-called congenital cases, in food idiosyncrasy, hay fever, etc., at considerable length because we wish to be just to all circumstances, and because such a view has been apparently accepted by investigators as experienced as Doerr, Coca and others. We still believe, however, that fundamentally such so-called congenital cases will probably be found to be very closely related to the acquired ones. We may, and experimental and clinical observations force us to admit, that there is an inheritance factor in hypersusceptibility. On the other hand, it is by no means impossible in our opinion that such an inheritance factor may consist largely in the bioplastic inheritance merely of a capacity for being easily sensitized. Such individual variations in reaction capacity to antigens

could be well assumed in human beings in whom inherited biological differences seem to be more common than in lower animals, such as guinea pigs. Such a simple inherited capacity for sensitization, therefore, would not only be possible, but likely; would then, of course, follow the general laws of inheritance, and would probably signify the inheritance of a capacity for antibody formation. Even in animals, as we have mentioned above, individually varied capacity for antibody formation is noticeable. Granted such an inherited predisposition to sensitization in general, it is still possible that every individual that actually becomes sensitive, does so only after preliminary contact with an antigen. Such a view would explain why the sensitiveness in the offspring is often for substances, different from those to which his ancestors were susceptible; and we have seen in analyzing the work of Cooke and Vander Veer, that, in the large majority of cases, hypersusceptibility does not develop until at least a year after birth. In such cases, granting the possibility of gradual sensitization through mucous membranes of the upper respiratory and intestinal canal, it would seem to us next to impossible to exclude antigenic contact; and in those cases in which sensitiveness develops very soon after birth, and actual personal contact can be excluded, prenatal sensitization through the mother, or a similar process during the period of lactation, is not entirely out of question in view of the work which has shown the possibility of the passage of proteins and antibodies through the placenta and perhaps through the milk. Let us not forget, moreover, that in the human species there is only one layer of cells intervening between the maternal and the fetal blood.[29]

Of importance is the question of passive transfer of these conditions. From the experiments of Schloss cited above, we have seen that in guinea pigs sensitized by mouth with the various foodstuffs, passive transfer was possible, and in some of his cases, as well as those of others, the serum of food idiosyncratic individuals conveyed specific hypersusceptibility to guinea pigs. As Doerr points out, however, this is insufficient to prove the true anaphylactic nature of the condition since it does not indicate definitely that the antibodies to the protein are involved in the process in the individual himself. We believe that perhaps this is an exaggerated point of view, but, nevertheless, it is, strictly speaking, logical. The transfer of the idiosyncrasy from one human being passively to another (the crucial experiment) is naturally a process for which the available literature is small. Ramirez, however, has published such a case in which a large amount of the serum of a horse-sensitive man was transfused to another individual who became typically horse sensitive, and developed cutaneous hypersusceptibility.

[29] Grosser. "Eihäute und der Placenta," Braumüller, Wien u. Leipzig, 1909.

Desensitization in these cases may be successfully carried out by a number of ways, according to pediatricians who have studied it. Schloss states that he has completely immunized three egg sensitive patients with injections of ovomucoid, starting with 0.0001 mg. of the ovomucoid in saline solution, and injecting it at intervals of 5 days, doubling the injection with each dose. He found a parallelism between the acquisition of immunity and the fading of cutaneous reactions. In one of his patients therapeutic injection caused moderate general symptoms in addition to a local reaction. A considerable number of his egg and milk cases he improved by gradually increased feedings of the responsible protein. The initial doses as given by him in capsule form were extremely small, namely, amounts of 2 to 5 mg. diluted in milk sugar or starch. Increases of dose were gauged by the results, and by the age of the patient. One infant, one year of age, was so sensitive to milk that swelling of the tongue and lips resulted from one drop of milk diluted with water. He started, in this case, with the administration of 1/20 of a drop three times a day.

Thus, again analogous with anaphylaxis, it seems possible to desensitize specifically in these cases, but we must remember, as repeatedly stated by Schloss and other pediatricians, that once desensitized, the ingestion of the responsible food must be continued, otherwise, again in analogy with anaphylaxis, the sensitive condition will reappear.

Before going on to hay fever, we may, therefore, summarize the so-called food idiosyncrasies by stating that here we have a series of conditions in which close analogy to anaphylaxis exists in the antigenic nature of the inciting substance, and in the existence of well-defined cases in which the condition was an acquired one. Additional corroborative analogy exists in experiments like those of Schloss, in which guinea pigs were sensitized by mouth. Moreover, specific desensitization further brings these cases into line with protein anaphylaxis. However, in these conditions the inheritance factor is a very distinct one, and separates them, in this respect at least, from true protein anaphylaxis; also the dependence of the condition upon specific antibodies has not been sufficiently demonstrated.

Hay Fever and Asthma.—The peculiar conditions which are grouped together under the term of "hay fever" were first associated with the inhalation of pollen by Blackley [30] as early as 1873.[31] Blackley also recognized a peculiar urticarial skin reaction when the material was rubbed upon the scarified epidermis, and observed catarrhal reactions upon experimental instillation of pollen upon the mucous

[30] Blackley. Cited from Longcope, *loc. cit.* His original work in "Experimental Researches on the Cause and Nature of Hay Fever," London, 1873.

[31] For historical literature see Cooke and Vander Veer, *loc. cit.*

membrane of the nose, and on the conjunctivæ. The supposition of Blackley concerning the relationship of pollen grains to the condition, was very thoroughly confirmed by Dunbar [32] who regarded the constituent in the pollen responsible for the reactions as primarily toxic. This idea has been pretty well eliminated, however, by subsequent researches, and it is quite definitely established that the disease is due to a specific hypersensitiveness to some material in the pollen and analogous to other forms of idiosyncrasy. According to Scheppegrell,[33] it has been estimated that in most parts of the United States from 1 to 2 per cent. of the population suffer from the disease at some period of the year. A considerable variety of plant pollens may be responsible. There was at first considerable difficulty in identifying the various pollen weeds which caused the disease. This was due, according to the writer quoted above, to the fact that many pollens are wind-borne, and often a common weed like the golden rod, because of its profusion in the neighborhood of the patient, was held responsible, in spite of the difficulty with which its pollen grains are distributed, while the more commonly responsible rag-weed was neglected. Scheppegrell characterizes hay fever weeds particularly as those which are wind-pollinated, are very numerous, with inconspicuous flowers, without bright color, or scent, in which pollen is formed in great quantities. All such weeds, he says, are suspicious from a hay fever point of view. The most common of these are the common rag-weed, and the giant rag-weed, in both of which the pollen is wind-borne, and is produced in such abundance that very slight winds will dislodge them and carry them in clouds to considerable distances. According to investigations cited by Scheppegrell, these weeds are responsible for probably 85 per cent. of all cases of autumnal hay fever in the United States. Many grasses, however, can also give rise to hay fever. For a botanical survey of these, we refer the reader to Scheppegrell.

People who are susceptible to hay fever are usually attacked in the autumn, or the summer at a time when the particular weed to which they are sensitive has reached the pollen stage. The disease usually appears gradually, with the general signs of a cold in the head, with itching and burning of the conjunctiva, and a watery catarrhal discharge from the nasal passages. In rare cases there may be fever and general signs of systemic malaise, fatigue and depression. While autumnal cases are the most common, this is not by any means the only season of the year in which the disease may occur, but as one would expect from the variety of plants which may be responsible, there are cases of so-called "rose-cold" which may occur

[32] Dunbar. *Deut. med. Woch.*, 29, 1903, p. 149.

[33] Scheppegrell. "Hay Fever, etc.," *U. S. Pub. Health. Rep.*, Rep. No. 349, July, 1916.

in the summer, and other cases are characterized by onset in the spring of the year.

In view of the minute dosage of responsible pollen material which causes violent symptoms in the susceptible individual, as compared with the apparently complete harmlessness of considerable quantities of the same material for normal individuals, it was quite natural that, as Wolf-Eisner [34] first suggested, that anaphylaxis-like hypersensitiveness should be considered as a possible mechanism in explanation of the condition. In consequence of this, many observers tried to produce artificial sensitization and antibodies in animals by injection of various pollen preparations. This has been attempted, especially by Cooke, Flood and Coca who failed entirely in attempts to produce precipitating and complement fixing antibodies by the injection of pollen extracts into rabbits, nor could they induce anaphylactic conditions in guinea pigs by sensitization with the pollen extracts. This failure to show antigenic properties is taken by Cooke and his associates, and by Coca, as one of the arguments on which they definitely rule hay fever out of the category of anaphylactic phenomena. As far as this particular point is concerned, experiments in our own laboratory, undertaken by J. T. Parker [35] because of this claim that pollen was not antigenic, definitely contradict the observations of Cooke and others. Because of their failures, the more delicate method of testing for anaphylactic sensitization by means of the Dale method was undertaken. Guinea pigs were sensitized by injection preparations of high rag-weed pollen extracted, with weak sodium hydrate and injected in twelve doses on successive days. The fluid, which gave Millon and Xanthroproteic tests, was given in large amounts to guinea pigs, a total of 70 c. c. being administered in the course of 7 weeks. This method was undertaken because in our work on sensitization with bacterial proteins, we had found it advantageous. For the same reason, injections were given every day for several weeks. About a month later, the uteri were tested and found highly and specifically susceptible to the extracts. We were careful in the publication of this research from our laboratory, to state that we did not believe that this proved the anaphylactic nature of hay fever, but the experiments were so clean-cut and unquestionable, that we have no hesitation in at least concluding from them that hay fever pollen does contain an antigenic substance. It is important, however, in this case, as we have found in our work on bacterial antigens which will be mentioned later, that sensitization of the guinea pigs follow the painstaking method we used, and that the production of extracts, if extracts are used, shall be made with originally alkalin solutions.

As to other work which may possibly indicate the intervention

[34] Wolf-Eisner. "Das Heufieber," Munich, 1906.
[35] Parker, J. T. *Proc. Soc. Exper. Biol. & Med.,* 18, 1921, p. 237.

of antibodies in the hay fever complex, there are isolated claims in which the negative results of Cook and his associates are contradicted. Clowes [36] reported the detection of precipitins and complement fixing antibodies in the sera of patients, and Koessler [37] claims that he passively sensitized guinea pigs to rag-weed pollen with the blood of a hay fever patient.

The fact that hay fever pollen does contain antigenic substances, together with the work cited above, does not, of course, prove the anaphylactic nature of hay fever which we, ourselves, consider uncertain; but these facts should not be completely thrown out of court in the discussion of hay fever, as this is done by Coca.

In hay fever, as in other conditions discussed, specific hypersensitiveness of the skin and of the conjunctiva run parallel with the degree of sensitiveness to the disease, and has been used by all observers who have studied the disease as an index for the diagnosis of the specific pollen responsible, and as an index for treatment. Vander Veer and Cooke recommend the intradermal injection of 1/50 to 1/100 of a cubic centimeter of a properly prepared pollen extract which in positive cases results within 5 to 20 minutes in a urticarial wheal, surrounded by a hyperemic zone from 1 to 3 cubic centimeters in diameter. This is usually accompanied by local itching. Their pollen extract consists of a simple extraction of the pollen with 0.8 per cent. sodium chloride solution, and are standardized into three strengths, according to the amount of nitrogen per cubic centimeter. They use three solutions, one containing 0.01 mg. of nitrogen, another with 0.1 mg., and a third with 0.5 mg. per cubic centimeter.

They first determine by intradermal test which pollen the individual is susceptible to. Then by the ophthalmic reaction, using the weakest solution first, they test the degree of sensitiveness. When a positive reaction is obtained with a solution containing 0.01 mg. per cubic centimeter, they give their first dose in an amount of 0.005 mg. of nitrogen per cubic centimeter. For a discussion of the treatment and its results, we refer the reader to the excellent article of Cooke and Vander Veer. From the point of view of the theoretical conception of the condition, the important matter is that consistent treatment with the specific pollen does desensitize the patients. The results have been so favorable that some improvement may be expected in 90 per cent. of the cases of real hay fever. The late hay fever cases, however, they state are much more difficult. As in other cases of desensitization, the effects are temporary, the desensitization, according to Freeman [38] rarely extending completely from one season to the other, and hardly ever to the second season.

[36] Clowes. *Proc. Soc. Exper. Biol. & Med.*, 10, 1913, p. 69. Cited from Cooke and Vander Veer.

[37] Koessler. "Forcheimer's Therapeusis, etc.," Vol. 5, p. 671.

[38] Freeman. *Lancet*, September, 1911; April, 1914.

While, therefore, we must again call attention to the important difference between true anaphylaxis and hay fever because of the probable rôle of inheritance, we can also emphasize the many points of definite analogy between the two conditions. There is an antigenic substance in the pollen, though it is not easy to demonstrate. Actual presence of antibodies in the blood of the patients, and the possibility of passively sensitizing with such blood, we regard as questionable, and still calling for further investigation. Phenomena of desensitization are analogous to those prevailing in anaphylaxis.

Drug Idiosyncrasies.—Changed reaction of the body to various drugs, alkaloids, glucosides, organic compounds, such as many hypnotics, to salicylic acid, and even to such simple substances as iodin and the iodides, arsenic, bromides, mercury, etc., has been a curious phenomenon that has puzzled investigators for a good many years. Acquired resistance to drugs, so-called drug tolerance has been known for a long time, and every physician knows, especially in the case of the alkaloids, the enormous quantities of highly toxic substances that can be supported by individuals who habitually use them. The same is true even of such simple substances as arsenic. In a preceding section dealing with immunization, we have discussed this problem briefly, the brevity of our treatment of the subject being enforced, in spite of its importance, by the incompleteness of our knowledge. Since all of these materials are non-antigenic in themselves and, therefore, develop no antibodies, since all efforts to demonstrate neutralizing substances in the blood of the tolerant individuals have so far been unsuccessful, and since there is almost complete unanimity as to the impossibility of transferring drug tolerance from one individual to another, there are very few experimental data from which theory can take its departure. The only thing that seems definitely established is that drug tolerance is a function of the tissue cells, rather than of any mechanism comparable to antibody formation. Just as there exists a drug tolerance which is to some extent analogous to immunity to antigens, so is there a changed reaction capacity in the opposite direction, namely, a drug hypersusceptibility, a class of conditions grouped together under the term of drug idiosyncrasy.

The earliest observations on drug idiosyncrasies were made largely by skin specialists who noticed peculiar forms of dermatitis and urticaria in connection with the administration of certain drugs. Jadasohn [39] was perhaps one of the first who systematically studied these phenomena, which he spoke of as "Arzneidermatosen," and which he classified into two groups of cases, one group in which the individuals react abnormally to the very first contact with the drug, and others in which repeated administration is necessary to develop the idiosyncratic susceptibility. The supposedly "acquired" cases seem to be relatively rare and in most cases the idiosyncrasy becomes

[39] Jadasohn. Quoted from Sauerland, *Berl. klin. Woch.*, 49, 1912, p. 629.

apparent without preceding habitual use. This is perhaps one of the most important arguments against classification with anaphylactic phenomena. Attempts to induce drug hypersusceptibility artificially in animals and in man have been generally unsuccessful as far as we can analyze the literature, although there are a certain number of cases in which salvarsan and quinin therapy have seemed to bring about hypersusceptibility.

The skin eruptions are not the only manifestations of this condition. Fever, systemic symptoms and a variety of other disturbances may characterize the attacks. It is important to note in this connection that the symptoms of the hypersusceptibility are not those of an increased development of the physiological action of the drug, but take forms quite easily distinguishable from the ordinary pharmacological action. Furthermore, the specific clinical pictures for one drug may be consistent and different from that induced by other drugs.

The problem is a complicated one, and calls for a considerable amount of investigation. The relationship of the sensitiveness to the chemical constitution of the drug is also a peculiar one. In some cases it is specific for the entire molecule, such as the idiosyncrasies occasionally noticed against some of the hypnotics. Bruck [40] reports a case of apparently acquired antipyrin susceptibility in a physician, who, after a considerable series of antipyrin administrations, began to suffer from aphthous eruptions of the tongue which always returned when he took antipyrin in the course of ten years. All antipyrin derivatives, pyramidon, etc., had the same effect. In other cases, such as iodoform hypersusceptibility, the hypersusceptibility seems to be specific for the methyl radical since, as Doerr states, such individuals react also to bromoform, etc. Doerr, however, also mentions cases such as quinin idiosyncrasies in which closely related substances gave no reaction whatever.

A great many investigations have been carried out with the purpose of bringing drug idiosyncrasies into close analogy with anaphylaxis. Such are the investigations of Bruck, Klausner [41] and others. Their experiments have consisted chiefly in attempting to transfer the sensitiveness of man, passively, to animals. Bruck injected guinea pigs with 5 c. c. of the serum of the antipyrin case spoken of above, and 24 hours later administered 0.3 grams of antipyrin subcutaneously. About three-quarters of an hour after the antipyrin injection, one of the guinea pigs that was supposedly passively sensitized, showed uneasiness, dyspnea and finally cramps and paralysis of the hind legs. It died 5 hours later. In criticizing this apparently clear experiment, it should be borne in mind that the amount of antipyrin administered was only 25 per cent. less than the

[40] Bruck. *Berl. klin. Woch.*, 47, 1910, p. 517.
[41] Klausner. *Münch. med. Woch.*, 27, 1910.

minimum toxic dose for a normal pig, and that of two pigs sensitized in exactly the same way, only one reacted. Cruveilhier [42] similarly claims to have produced antipyrin hypersusceptibility, both actively and passively, in guinea pigs. With these and a few other exceptions, all other attempts in a similar direction seem to have been negative. Auer,[43] encouraged by the observation that repeated injections of salvarsan occasionally give rise to anaphylaxis-like reactions [44] in man, attempted to throw light upon this condition by animal experiments. He injected salvarsan, both in acid and alkalin solutions in concentrations of 0.1 per cent., into guinea pigs and rabbits. In none of them was he able to produce any degree of hypersusceptibility.

It will be seen from the foregoing that our knowledge of the laws governing idiosyncrasies is defective and available evidence is contradictory. There is very little therefore on which reliable opinion can be based. The only line of research which has shown possible promise of eventual light upon the subject is that initiated by Obermeyer and Pick,[45] and recently further developed by Landsteiner.[46] Obermeyer and Pick, as we have seen in another part of this book, treated proteins with various chemical substances such as Lugol's solution, and succeeded in so altering the serum protein that it changed in specificity without losing its antigenic properties. Thus, an iodized protein now acted as antigen with sera produced against many other iodized proteins, the insertion of the iodin atom in the serum apparently having changed its specific antigenic nature. Landsteiner has done exceedingly interesting work along similar lines in azotyzing various proteins by treating them with metanilic acid (an amino-sulphone-benzoic acid), and immunizing rabbits with the altered protein. The antibody produced by the rabbits now precipitated only azo-proteins, and not the native proteins from which the azo-protein had been produced. Apparently, the specificity of an antigen, therefore, may be considerably altered by coupling it with other chemical groups, the added group determining the specificity. Wolf-Eisner has suggested this as an explanation for many drug idiosyncrasies, assuming that the administered drug may in some way form a compound with the serum protein, permitting the final product to react within the body as a foreign antigen. Swift [47] working along this line of reasoning, treated guinea pigs with salvarsanized guinea pig serum. With this mixture he sensitized guinea pigs and obtained a few reactions which might be interpreted as truly anaphylactic in nature. He naturally expressed himself in a very conservative manner.

[42] Cruveilhier. *Comp. rend. d. l. Soc. Biol.*, 69, 1910.
[43] Auer. *Jour. Exp. Med.*, 14, 1911, p. 497.
[44] Weischelman. *Deut. med. Woch.*, 1912, p. 1174.
[45] Obermeyer and Pick.
[46] Landsteiner. See section on Antigens.
[47] Swift. *Jour. A. M. A.*, 59, 1912, p. 1236.

While this general direction of research is attractive, it cannot be said to have so far had very much effect upon our conception of drug allergies, largely because so many contradictions prevail in the literature. We do not feel at the present time that any concise opinion concerning the nature of drug allergies or their relation to anaphylaxis is warranted. All we know is that we have here both tolerance and hypersusceptibility, in many ways analogous to immunity, on the one hand, and anaphylaxis on the other; but since the inciting substances are diffusible and can easily penetrate into the cells, no systematic investigation of the mechanism has been possible. It may well be that the reasoning of Swift based on such unquestionably definite experiments as those of Landsteiner may explain certain types of acquired drug allergies. The remainder in which the process is probably intracellular, must await further investigation.

It is worth noting, moreover, that in most instances in which passive transfer of a drug idiosyncrasy has been claimed, the drug employed has been one of those with which human beings occasionally acquired their hypersusceptibility as a consequence of habitual use. Thus, Bruck's case was one of antipyrin idiosyncrasy in a physician who did not develop it until he had been taking the drug for a long time. With the serum of this patient he claims to have passively sensitized guinea pigs. Cruveilhier's work was done with antipyrin, and Swift's with salvarsan, and in the case of salvarsan, not only have we definite records of occasional cases in which severe reaction followed after several harmless preliminary injections, but we have a number of reasons to believe that the drug undergoes some form of combination with the serum. This is apparent from the work of Swift and Ellis on the use of salvarsanized serum in spinal cases, and also from the fact that the treponemacidal action of salvarsan is not a function of the pure solution, in which the treponemata do not seem to be particularly injured, but becomes evident only when serum is present. Thus, it is not at all out of question that drug idiosyncrasies are not all alike, and although most of them are so far inexplicable on the basis of pure analogy with anaphylaxis, there may be others which depend upon the mechanism of union between the drug and the serum constituents, thereby producing an altered antigen within the body. And this possibility becomes particularly important in view of the work of Landsteiner [48] in which he succeeded in altering rabbit sera by treatment with alcoholic solutions of sulphuric acid, so that an altered antigen resulted which would produce antibodies, even in the rabbits from which the original protein was obtained. The species specificity was destroyed, and a "sulphon" specificity produced which then acted on the same species from which the serum was derived.

[48] Landsteiner and Jablons. *Zeitschr. f. Imm. u. Exp. Ther.*, Vol. 20, 1914, p. 618.

CHAPTER XIX

BACTERIAL ANAPHYLAXIS. TUBERCULIN AND ALLIED REACTIONS. BACTERIAL ANAPHYLATOXIN THEORIES. TOXIN HYPERSUSCEPTIBILITY. TOXICITY OF NORMAL SERUM. GENERAL CORRELATION.

In the case of most serum reactions the original observations were made upon the sera of bacteria-immune animals, and later expanded into generalizations applicable to antigens as a class. This was the case with the phenomena of lysis, agglutination, and precipitation. In the case of anaphylaxis the reverse was true. The fundamental observations were made with non-bacterial antigens, but the thought that analogous observations could be made with bacterial proteins was an obvious one, and since the problem was one of altered susceptibility there was great promise that investigation of this subject might prove of profound significance for our knowledge of the pathology of infectious diseases.

Accordingly Rosenau and Anderson,[1] in one of their earliest researches, carried out experiments upon the sensitizing properties of bacterial proteins. They were successful in sensitizing guinea pigs with extracts of colon, tubercle, anthrax, and typhoid bacilli, with *Bacillus subtilis* extracts, and with those of yeast. In most cases they used considerable quantities of bacterial extracts and obtained but slight or moderate symptoms. However, their results were conclusive in showing that the anaphylactic experiment could be carried out with bacterial proteins and was, in every detail, analogous to the similar phenomena of serum anaphylaxis.

Not only could the basic experiment of active sensitization be carried out with these substances, but it was found that the reaction here, as in other cases, was specific, and that shock was followed by a period of "antianaphylaxis" or "immunity." Rosenau and Anderson suggested that the incubation time of many infectious diseases may be represented by the period necessary for the development of susceptibility after a first injection, and that the crisis of pneumonia might possibly find an explanation in the analogy with anaphylaxis.

The criteria governing the successful production of bacterial anaphylaxis were then studied especially by Kraus and Doerr,[2] Holo-

[1] Rosenau and Anderson. *U. S. Pub. Health and M. H. S. Hyg. Lab. Bull.* 36, 1907.

[2] Kraus and Doerr. *Wien. klin. Woch.*, No. 28, 1908.

but,[3] Delanoë[4] and others, and the essential points of Rosenau and Anderson's experiments were confirmed. Although Kraus and Doerr succeeded in frequently sensitizing guinea pigs with a single injection of bacteria, this was not found to be the most favorable method for sensitization. Braun [5] obtained entirely negative results by such a procedure, but this may well have been because in the first place single sensitization with bacteria is evidently irregular in result, and because Braun carried out his intravenous test-injection *slowly,* a technique by which Friedberger found later that shock could be avoided. Delanoë, in the main, confirmed the fact that bacterial sensitization was possible, but denied the specificity of the resulting anaphylaxis, in that he succeeded in producing shock in tubercle-sensitized guinea pigs with comparatively large amounts of typhoid, paratyphoid, and other bacilli, and conversely found typhoid-sensitized guinea pigs hypersusceptible to tubercle-injections. Other workers, however, notably Kraus and Doerr, Holobut, and Kraus and Admiradzibi,[6] agree that the reaction is specific, at least in the same limits within which other serum reactions may be called specific.

Holobut then developed a technique of sensitization with bacteria more reliable than any which had been previously employed by other workers. He found that the most regularly successful results were obtained when he injected small quantities of bacteria (1/100 loopful) daily for ten days, subcutaneously, and tested with fairly large amounts (1-2 c. c. of an emulsion of the bacteria) intravenously about 3 weeks after the last sensitizing injection. This is in keeping with later experience, and in our own work with typhoid immunization in young goats we have found that anaphylactic reactions were not observed unless the goats had previously received several injections. A second injection never elicited symptoms.

It is hardly worth while to recount all the various methods recommended for the successful sensitization of guinea pigs and other animals with bacterial proteins. The general principle that it is not as easy to sensitize with bacterial substances as with proteins, like egg-white and the various animal sera, may be regarded as well established. In our own work with tubercle bacillus protein we attained a considerable regularity of results by injecting ten and twelve times intraperitoneally either daily or every other day and testing between two and three weeks after the last injection. Quantities used for injection were considerable, consisting of one or more cubic centimeters of a suspension or extract of ground dead tubercle bacilli.

[3] Holobut. *Zeitschr. f. Immunitätsforsch.,* Vol. 3, 1909.

[4] Delanoë. *C. R. de la Soc. de Biol.,* Vol. 66, 1909, pp. 207, 252, 348, 389.

[5] Braun. Quoted by Bail and Weil, *Zeitschr. f. Immunitätsforsch.,* Vol. 4, 1910.

[6] Kraus u. Admiradzibi. *Zeitschr. f. Immunitätsforsch.,* Vol. 4, 1910.

Thus, while active sensitization is perfectly definite in the case of bacterial materials, care and attention to this principle is necessary in demonstrating it. The reasons for this are probably to be found in the constitution of the bacterial protein. The bacterial cell body contains a relatively small amount of coagulable albumin or globulin like substances, and it is, therefore, necessary to administer very considerable amounts of bacterial material in order to simulate anything like the quantities of coagulable protein obtained even in small amounts of serum. Moreover, it is not at all impossible that most of the bacterial substances ordinarily injected are not in solution, and are actively phagocited with consequent loss of antigenic stimulation of the body cells. The major part of the bacterial cell bodies seems to consist of nucleo-protein-like materials, and a residue which will be described below, the chemical nature of which is not as yet quite certain. This residue is antigenic in the sense that it reacts with antibodies, but it seems to be either extremely difficult, or impossible to incite antibody formation in the animal body by injecting it. These substances which we have been studying for some time, probably represent bodies like the "Haptenes" foretold by Landsteiner. It seems plain, then, that the most likely explanation for the difficulties one encounters in sensitizing with bacterial proteins is due to the peculiar chemical constitution of the bacterial cell body. A small amount of Bence-Jones protein has been found in bacteria by us in our tuberculin studies, and Bence-Jones protein has been found by Bayne-Jones to have antigenic properties. The antigenic constitution of the bacterial cell, then, is a complex one, and this must be taken into consideration when drawing conclusions about anaphylaxis or other immunological reactions against bacteria.

Passive sensitization against bacterial substances has also been obtained, in all respects analogous to the passive sensitization produced in serum anaphylaxis. The earlier work on this was done by Kraus and Doerr,[7] and by Kraus and Admiridzibi,[8] and their findings have been variously confirmed by Holobut,[9] Yamanouchi,[10] Delanoë [11] and others. Some of these writers, especially Yamanouchi and Delanoë, suggested the utilization of passive anaphylaxis for the diagnosis of tuberculosis and typhoid fever. However, their experiments were entirely too indistinct to warrant such procedures, and even now that we know more about passive sensitization, the method would be of little practical usefulness.

In order to eliminate any possible sources of misinterpretation, the writer and Parker [12] some years ago reinvestigated passive sen-

[7] Kraus and Doerr. *Wien. klin. Woch.*, 21, 1918, p. 1008.
[8] Kraus and Admiridzibi. *Zeitschr. f. Immunitäts.*, Orig., 4, 1909, p. 607.
[9] Holobut. *Zeitschr. f. Immunitäts.*, 3, 1909, p. 639.
[10] Yamanouchi. *Compt. Rend. d. l. Soc. Biol.*, 66, 1909, p. 531.
[11] Delanoë. *Compt. Rend. d. l. Soc. Biol.*, 66, 1909, pp. 207, 252, 348, 389.
[12] Zinsser and Parker. *Jour. Exp. Med.*, 1917, p. 411.

sitization of guinea pigs to bacteria by the isolated uterus method of Dale. Working with typhoid bacilli and young passively sensitized guinea pigs, it was found very easy to sensitize these animals with 1 c. c. of anti-typhoid serum of an agglutinating titre of about 1-10,000, the sensitiveness developing a little more slowly than in the experiments reported by Kraus and his collaborators, being most thoroughly developed after about 3 to 5 days. At this time typical shock could be elicited in some of the animals by intravenous injection of a cubic centimeter of simple extracts of typhoid bacilli, and typical uterine contraction could always be obtained by the Dale method.

These experiments also enabled us to investigate the extremely interesting question raised by Friedberger's anaphylatoxin work, namely, whether typical anaphylactic reactions in guinea pigs could be produced by the intravascular production of toxic substances from the bacteria. It opened the problem of the mechanism by which organisms like the typhoid bacillus intoxicate an animal, and whether or not this has any relation to anaphylaxis. Our experiments showed the following points, to which we will refer later in connection with the discussion of Friedberger's anaphylatoxin work. In the first place, it was found that the normal unsensitized guinea pig uterus showed no anaphylactic contractions when brought into contact with considerable amounts of typhoid extract. This would mean that the anaphylactic mechanism of injury does not enter into the acute death of unsensitized guinea pigs into which one injects large quantities of dead or living typhoid bacilli sufficient to kill them in a few hours. The mechanism of injury here must depend upon some toxic product probably formed intravascularly in which anaphylaxis-like processes, and probably antibodies, play no rôle; it may be due to the liberation of endotoxin-like substances in the sense of Pfeiffer, or a toxic split produce in the sense of Friedberger's interpretation. The uteri of sensitized guinea pigs, however, are delicately susceptible to similar extracts, showing that in animals or human beings in which typhoid bacilli have been present long enough to give rise to antibody production, the anaphylactic mechanism of injury may be superadded to the other. It is, furthermore, we think, of considerable interest that the sensitized uterus, though delicately susceptible to the extracts of typhoid bacillus, was hardly, or not at all affected by whole washed typhoid bacilli. In other words, in order to give rise to anaphylactic symptoms, the bacterial antigen must be in solution, and such processes can take place only in the infected animal body as there is a destruction of the morphological integrity of the bacterial cell in the circulation. Rapid phagocytosis would, therefore, protect against it. Moreover, these experiments demonstrated the essential cellular nature of bacterial anaphylaxis.

It is, thus, plain that all the analogies with serum and protein

anaphylaxis have been fulfilled with bacterial materials, and that if this particular mechanism plays any part in infectious disease, it must be under conditions in which sessile antibody formation has taken place and the bacterial protein has gone into solution in some way in the body.

There is, however another form of hypersensitiveness in the animal body to bacterial products about which there has been a considerable amount of discussion, and the relationship of which to true protein anaphylaxis is not entirely clear. This is the type of hypersensitiveness exemplified by the tuberculin, typhoidin, etc., reactions.

Tuberculin and Similar Reactions.—There is one class of phenomena, however, which calls for further discussion in this connection, since its dependence upon anaphylaxis, while generally assumed, is still opposed by many authorities. This consists of the various REACTIONS in which micro-organisms are injected, or brought into contact with the skin or conjunctiva of infected subjects. Such are the various forms of the *tuberculin reaction,* the *typhoidin reaction* of Chantemesse, the one of Gay, and the mallein and abortin reactions.[13] In the tuberculin reaction the conditions have been thoroughly studied, and we may make a detailed consideration of this example serve to bring out the general principles involved.

In all forms of the TUBERCULIN REACTION there is a very evident hypersusceptibility to various forms of antigen derived from the bacillus. When the tuberculin is injected subcutaneously the reaction is systemic and also localized to a certain extent in any tuberculous foci which may be present. When the v. Pirquet or Moro skin reactions are carried out, or the Calmette ophthalmic test is made, the reactions are almost purely local. In all cases reactions are induced by quantities of antigen which cause no effect whatever in normal individuals.

The basic observation leading to the diagnostic use of tuberculin was made by Koch [14] upon guinea pigs. He describes his observation as follows:

"Tuberculin may be injected into normal guinea pigs in considerable quantities without causing noticeable symptoms. Tuberculous guinea pigs, on the other hand, react to comparatively small doses in a very characteristic manner."

Since, in Koch's experiments upon tuberculin, it was desirable for his particular purposes at the time to obtain very sharp reactions, he did not content himself with the production of moderate symptoms by the injection of slight amounts of tuberculin into in-

[13] We do not include Noguchi's luetin reaction since we do not believe that this reaction is specific. Our original view on this, based on studies of culture treponemaba, has been borne out by clinical studies. See Kolmer & Greenbaum, *Jour. A. M. A.*, 79, 1922, p. 2063.

[14] Koch. *Deutsche med. Woch.*, No. 43, 1891.

fected animals, but increased his dosage until the guinea pigs were killed. He showed that guinea pigs having a moderately advanced infection—4 to 5 weeks after inoculation—could be killed by doses of 0.2 to 0.3 gram, while animals in very advanced stages would succumb within 6 to 30 hours to quantities as small as 0.1 gram subcutaneously. In the animals so studied he determined not only a systemic effect, but a very marked local reaction as well in the skin, areolar tissues, and adjacent lymph nodes.

Koch's observations upon guinea pigs were applied by him, Guttstadt,[15] Beck,[16] and others to man, and the result was the development of the present important diagnostic test. The fundamental fact in this as well as in other tests of this kind, then, is the appearance of local and systemic reactions in infected subjects to contact with specific antigenic material which, at least in the same quantities, produces no effects in normal individuals. The analogy with the phenomena of anaphylaxis is thus indicated.

Koch's original interpretation of the phenomenon was of course unaided by any of the later observations on anaphylaxis. According to him the tuberculin contained substances which caused tissue necrosis. The necrotizing action was particularly powerful upon tissues which were tuberculous, and therefore already saturated with the toxic material. The destruction of such tissues resulted in systemic symptoms.

Very similar to this view is the one later expressed by Babes and Broca,[17] who attribute the systemic symptoms to a sudden lighting up of the existing lesions by the small amount of extra tuberculin added to that already present in these foci.

The first suggestion of the possible association of the tuberculin reaction with the union of an antigen and its antibody was made by Wassermann and Bruck.[18] They accepted Ehrlich's assumption that certain cells of the tuberculous foci (those situated just below the periphery and already affected by the tubercle toxin, though still resistant) were possessed of an increased receptor apparatus for the tubercle antigen. For this reason the injected tuberculin was concentrated in these foci, attracted out of the circulation by the increased avidity of these cells, the consequence being increased activity of the lesions and systemic symptoms. The tuberculin reaction, according to these writers, therefore, would be caused by the union of the tuberculin with the "sessile receptors" upon the diseased tissues—a point of view which would specify the diseased tissues and their products as the sources from which emanated the toxic factors inciting the systemic symptoms.

[15] Guttstadt. "Klin. Jahrbuch" Ergänzungsband, 1891.
[16] Beck. *Deutsche med. Woch.*, No. 9, 1899.
[17] Babes u. Broca. *Zeitschr. f. Hyg.*, Vol. 23, 1896.
[18] Wassermann and Bruck. *Deutsche med. Woch.*, No. 12, 1906.

The theories of Koch and of Babes do not, as Meyer points out, explain the frequent absence of the tuberculin reaction in very advanced cases of human tuberculosis, as contrasted with its frequency and regularity in the earlier cases. For, according to both of these views, the more severe the existing lesions the more actively would the injected tuberculin initiate tissue necrosis and consequent symptoms. The theory of Wassermann and Bruck avoids this objection since it presupposes the acceptance of Ehrlich's view that the increased receptor apparatus is present and free only in those cells in which necrotic destruction has not yet set in. In the necrotic areas the receptor apparatus is already saturated or satisfied as to its affinities, and extensive areas of necrosis, therefore, are unaffected by contact with further quantities of tuberculin.

These theories have been mentioned for the purpose of completeness, but can be dismissed at the present time as no longer fitting in with more recent observations on this type of reaction. The most important advances made in the general understanding of reactions of this type have been made since the development of the so-called skin reactions by von Pirquet [19] and the opthalmo reactions, by Calmette [20] and by Wolff-Eisner.[21] Von Pirquet found that when a small amount of a 25 per cent. solution of old tuberculin was applied to a slightly scarified area on the forearm in tuberculous patients, a typical, now generally well known, reaction occurred. The actual diagnostic value of the reaction has lost considerable weight, since this discovery by the recognition that in human beings the test is such a delicate one that over 70 per cent. of adults react positively, and even in children above two years of age, a positive reaction does not by any means signify an active tuberculosis. The same is true to about the same extent of the opthalmo reaction which consisted in the instillation of a 1 per cent. solution (of an alcoholic precipitate of old tuberculin) in distilled water into the eye. However, that these various tuberculins are specifically related to infection with the tubercle bacillus has been subsequently worked out with considerable accuracy in animals. The form in which the reactions have been mainly done in recent years in man and in animals is by the intracutaneous injections of minute amounts of the respective substances.

Since similar facts were elicited in the case of other infections, such as glanders, typhoid, B. abortus, etc., it was apparent that we had here a specific hypersusceptibility of animals, and man, to a bacterial product which, though it differed in important respects from the phenomena of true protein anaphylaxis, nevertheless, manifested

[19] Von Pirquet. *Berl. klin. Woch.*, 20, 1907, p. 644 and 22, 1907, p. 699. "Klinische Studien über Vaccination," *Deuticke,* Wien, 1907.

[20] Calmette. *Compt. rend. d. l'Acad. de Scien.*, June, 1907.

[21] Wolff-Eisner. *Berl. klin. Woch.*, 1907, p. 1055, discussion of paper by Citron.

a great many analogies. Von Pirquet, accordingly, included the reaction under the phenomena of what he called allergy, or altered reaction capacity.

He assumed that the reaction depended upon the presence in the system of antibodies, which formed a union with the applied tuberculin, the result being the formation of poisons and a reaction. This assumption, according to the anaphylatoxin theory of Friedberger, would imply the participation of alexin—acting upon the united tuberculin-antituberculin complex, though v. Pirquet does not express himself positive as to this.

This, moreover, is the clearly expressed opinion of Friedberger.[22] Consistently with his general theory of anaphylaxis he assumes that the injected tuberculin comes into relation with specific antibodies with which it unites, the alexin then splitting off anaphylatoxin from the complex. He bases this view upon his experimental demonstration, mentioned above, of the production of anaphylatoxin from tubercle bacilli by *in vitro* digestion with guinea pig complement.

In principle the view of v. Pirquet is similar to that previously expressed by Wolff-Eisner [23] that the union of tuberculin with its lytic antibody, present in the tuberculous animal, gave rise to poisons as the result of lysis. Both of these theories simply apply, to the special case of the tuberculin reaction theories of mechanism applied by these workers to anaphylactic reactions in general.

Calmette,[24] who separates the tuberculin reactions distinctly from anaphylaxis, nevertheless assumes that tuberculous animals and man develop a lytic principle, presumably in the nature of a bacteriolysin, which enters into reaction with the specific antigen in the tuberculin preparations. This, again, is fundamentally an anaphylatoxin view in Friedberger's sense.

However, more recent careful analysis of tuberculin and similar reactions in animals have yielded a great many important facts which show that while there is much fundamental analogy between these reactions and anaphylaxis, there are, on the other hand, also, important differences which will not permit us to assume exactly the same mechanism in both.

Study of the reactions from a theoretical point of view was begun by Römer [25] in 1909, and by Baldwin [26] in 1910. Baldwin's work is fundamental, in showing that, in spite of previous assertions, guinea pigs could not be rendered skin-sensitive by implantation of porous filter capsules or celloidin capsules containing tuberculoprotein, or living tubercle bacilli. He showed that skin sensitiveness could never

[22] Friedberger. *Münch. med. Woch.*, Nos. 50 and 51, 1910.
[23] Wolff-Eisner. *Berl. klin. Woch.*, Nos. 42 and 44, 1904.
[24] Calmette. "L'infection bacillaire et la tuberculose," Paris, 1920.
[25] Römer. *Berl. klin. Woch.*, 46, 1909, p. 813, and *Deutsch. med. Woch.*, 35, 1909, p. 245.
[26] Baldwin. *Jour. Med. Res.*, 22, 1910, p. 189.

be produced without actual infection with living organisms. Animals treated with tuberculoprotein, however, often showed reactions to intravenous inoculation of the homologous preparation which could be recognized as anaphylactic. His conclusions can be summarized as follows: Tuberculous animals become sensitive to anaphylactic test, but not uniformly so. There is no absolute relation between the degree of sensitiveness and the stage of the disease. Injections of the tuberculoprotein may sensitize normal guinea pigs. Sensitized guinea pigs, however, do not react to the ordinary tuberculin test, though some respond slightly to the intradermal test. He adds: "This difference between anaphylactic sensitization and tuberculin reactivity need not be fundamental, as it is possibly due to another factor as yet undetermined." His experiments on the transfer of passive anaphylaxis to tuberculoprotein were inconclusive, but it has been shown since then, by Austrian [27] and others, that passive sensitization can be attained. From a theoretical point of view the most important observation of Baldwin is the fact that there seemed to be a discrepancy between skin sensitiveness and general anaphylaxis. Krause,[28] following out the work of Baldwin, confirmed and extended the above observations and established an interesting relationship between skin sensitiveness and the progress of infection. He asserts that skin sensitiveness develops simultaneously with the development of the initial focus, increases progressively with the lesions, varies directly with the extent and intensity of the infection, and diminishes with healing. It is blunted by a general tuberculin reaction which suggests analogy to saturation, such as that which occurs in connection with anaphylaxis.

In dealing theoretically with the tuberculin reaction, especially as regards the possibility of its being an anaphylactic phenomenon, it is of great importance to determine whether or not the reaction can be transmitted passively. The evidence on this is contradictory. Only a few writers have reported positively in this connection. In 1909, Bail [29] injected finely divided tissue mash of tuberculous organs of guinea pigs into normal guinea pigs, and 24 hours later gave the animals so treated 0.5 c. c. of old tuberculin, or a preparation which in this quantity had practically no effect upon normal animals. The animals prepared with the tuberculous tissue died in some cases, while controls treated with normal tissue suspensions showed no symptoms whatever.

Helmholz [30] in the same year reported positive skin reactions in normal guinea pigs 2 to 6 days after he had injected them intraperitoneally with the defibrinated blood of tuberculous guinea pigs.

[27] Austrian. *Bull. Johns Hopkins Hosp.*, 23, 1912, p. 1.
[28] Krause. *Amer. Rev. Tuberc.*, 1, 1917-1918, p. 65.
[29] Bail. *Zeitscher. f. Immunitäts.*, Orig., 4, 1909, p. 470.
[30] Helmholz. *Zeitschr. f. Immunitäts.*, Orig., 3, 1909, p. 371.

Both of these observations would be of fundamental importance if they could be confirmed.

In considering the mechanism of the tuberculin reaction, it will be well to examine also the work that has been done on the typhoidin reaction. Gay and Claypole [31] believed that positive skin reactions in rabbits were parallel with the degree of immunity of the animal. They succeeded in transferring the susceptibility to typhoidin from an immune to a normal animal by inoculation of 20 c. c. of typhoid-immune serum, testing 24 hours later. These experiments were repeated and confirmed by Meyer and Christiansen; [32] and in their first work with rabbits, these last observers, using what they called a typhoid autolysate (by which they mean an alcohol precipitate of a heated distilled water suspension of a 48 hour agar culture), concluded that "the typhoidin and similar reactions in rabbits are anaphylactic in nature and the result of an interaction of antigen and antibody." They stated that "the logical assumption from these facts is that cutaneous hypersensitiveness is the result of bacterial protein sensitization." Later Meyer [33] found that injected rabbits react with typhoidin more intensively than do immunized rabbits, and drew the conclusion that cutaneous hypersensitiveness does not indicate that the rabbits are particularly immune, and that no definite relationship existed between agglutinins and complement-fixing antibodies and skin sensitiveness. From these first two papers of Meyer's we gather that he believed that in rabbits skin sensitiveness to typhoidin is a sign of infection, rather than of immunity, but that as stated in his own words "cutaneous hypersensitiveness of rabbits . . . is, in all probability, the result of sensitization with typho- or similar bacterial proteins." Nichols [34] also considered the typhoidin reaction as he observed it in human beings as a protein sensitization.

The apparent discrepancies between the results of Baldwin with the tuberculin reaction and those of the workers just mentioned with the analogous typhoidin reaction, probably depend upon the fact that Baldwin used guinea pigs and the other observers used rabbits. When, subsequently, Fleischner, Meyer and Shaw [35] studied cutaneous hypersensitiveness in guinea pigs treated with repeated intraperitoneal injections of B. abortus, B. typhosus, and old tuberculin, and carried out parallel experiments with animals infected with living organisms the conclusions that they reached coincided with those of Baldwin in the case of the tuberculin reaction. They found, in other

[31] Gay and Claypole. *Arch. Inter. Med.*, 14, 1914, p. 671.

[32] Meyer and Christiansen. Collected Repr. G. W. Hooper Foundation for Med. Res., 2, 1916, p. 1.

[33] Meyer. Collected Reprints G. W. Hooper Foundation for Med. Res., 2, 1916, p. 68.

[34] Nichols. *Jour. Exp. Med.*, 22, 1915, p. 780.

[35] Fleischner, Meyer and Shaw. *Amer. Jour. Dis. Child.*, 18, 1919, p. 577.

words, that guinea pigs treated with dead bacterial proteins might become anaphylactic, but did not give skin reactions.

It will be seen, thus, that while results in rabbits are confusing, the conditions in guinea pigs are more or less analogous to those prevailing in human beings, and there seem to be at least two fundamental differences between protein anaphylaxis and reactions like the tuberculin reaction. On the one hand, these reactions, unlike true anaphylaxis, cannot be elicited by the treatment of animals with the dead bacterial products but occur only in animals that have been infected with the living micro-organisms. In the second place, passive transfer of typical tuberculin sensitiveness, in spite of the isolated observations to the contrary cited above, cannot be accepted as established.

Our own work [36] dealing with this matter confirmed these points, and was suggestive of a possible explanation. Studying particularly guinea pigs in regard to the relationship between skin reactions, anaphylaxis and infection, it was first found that, in these animals, two types of skin reaction could be elicited, just as this was possible in the human being. In typical protein anaphylaxis, with antigens like horse serum, during the period of sensitiveness, it was possible to elicit the urticaria-like, evanescent skin reactions noticeable in horse serum sensitiveness in human beings by the intracutaneous injection of small amounts of the antigen. When guinea pigs were treated for long periods with extracts of ground tubercle bacilli, they became typically anaphylactic as shown by the uterine reaction, but never developed typical tuberculin skin reactions. Such reactions, however, could always be developed within 8, 9, or 10 days in guinea pigs infected with living tubercle bacilli. Typical anaphylaxis, also, occurred in tubercle infected guinea pigs, but did not develop in less than 3 weeks or more; so that it was definitely possible to separate the typical, slowly developing and necrotic tuberculin skin reaction from true anaphylaxis, in that infected animals would show only the tuberculin reaction for considerable periods and no signs of uterine anaphylaxis; whereas, animals treated with dead materials would eventually develop true anaphylaxis but not skin reactiveness.

In following up these phenomena, it was found that the material which elicited the typical tuberculin reactions were chemically quite different from the coagulable proteins of the bacterial body, or of animal sera. The materials which gave typical tuberculin reactions (and only in isolated instances anaphylactic response in the uterus) were prepared as follows:

From powdered tubercle bacilli of the human type we produced extracts by shaking in a 0.02 per cent. sodium hydroxide solution in physiological salt solution. Three or four hours' shaking and perhaps a day or so in the ice chest sufficed to bring a considerable amount of the material into solution.

[36] Zinsser. *Jour. of Exp. Med.*, Vol. 34, 1921, p. 495.

This extract was centrifugalized until all the particles had been removed and a moderately opalescent supernatant fluid was decanted.

This material, upon being acidified to approximately Ph 5 to 6, with 2 per cent. acetic acid in the cold, became turbid and soon precipitated in large flakes. Further acidification up to Ph 4 did not redissolve these flakes. The precipitate represented the bulk of the dissolved substance in the extracts. The precipitate could be redissolved in a slightly alkalin salt solution and reprecipitated with acid. Because of their precipitability by acetic acid in the cold, we designated these substances as nucleoproteins or phosphoproteins, although we do not wish to commit ourselves, chemically, since we are aware of the indefiniteness of these biochemical terms and realize fully our incompetence to deal authoritatively with a chemical problem of such difficulty, without further intensive study.

After removal of these acid-precipitable substances by centrifugation and filtration through Berkefeld candles, the fluid was brought to a boil in the acid condition, and sometimes a very faint turbidity developed which was taken to represent the presence of coagulable protein, albumin or globulin, or both. These precipitates were so fine and slight that only Berkefeld filtration would remove them, and even this was not always completely successful.

The fluid was then neutralized; that is, brought to an approximate Ph of 7.

When this neutral fluid was filtered hot, we observed on numerous occasions that a slight precipitate developed over night in the ice chest, and that this precipitate redissolved on heating, a point which indicated the possibility that the tubercle bacilli may contain Bence-Jones protein. This point, however, will need further chemical analysis, a task which we have not yet had time to undertake. The water-clear fluid which was left after removal of all these substances gave in all cases a very definite precipitate with alcohol. The precipitate could be thrown down, collected and redissolved in water with salt solution, and like the similar precipitate obtained from crude tuberculin was astonishingly soluble.

This final water-clear material gave no biuret reaction, and usually gave no sulfosalicylic acid reaction, though occasionally this was very faint; in most cases it gave no Millon reaction, was not clouded on boiling with acid, and usually gave no xanthro-protein reaction, though in some cases a slightly yellowish color appeared when the ammonia was added in the second part of the test. This material, for want of a better name, we speak of as the "proteose" residue.

Materials of this kind have since been prepared by us from a number of other bacteria, and have been found to be excellent antigens in the sense that they react by precipitation and complement fixation with anti-sera; they have not so far produced antibodies after injection into animals, although extensive efforts to do this are still being carried on. It is not impossible that they represent the "Hoptenes" of Landsteiner. Whether or not they represent non-coagulable proteins—like some of the plant proteins studied by Wells, or whether they are molecularly simpler nitrogenous substances we cannot say as yet but hope to ascertain shortly.

Thus, to summarize the relation of reactions like the tuberculin reaction to anaphylaxis, the chief differences are the association of the tuberculin type of reaction with actual *infection* in which the body is constantly under the influence of substances diffusing out

from the growing focus; the quantitative and time factor of sensitization, therefore, is different. That the sensitization must be derived from a specific stimulus emanating from the bacteria seems unquestionable because of its specificity in each type of infection. Moreover, the failure of passive sensitization in these reactions, together with the more slowly developing and necrotic type of skin reaction, would influence one to consider that these reactions are intimately associated with the cell protoplasm, rather than merely with the cell surfaces. Differences in the chemistry of the inciting substance, namely, the probability that in the tuberculin and similarly prepared antigens from other bacteria, we may be dealing with a smaller molecule, certainly not with a coagulable protein, would make it seem possible that an important factor in these reactions might be found in differences of chemical structure, and perhaps of diffusibility between these antigens and the coagulable proteins which constitute the antigen in true anaphylaxis. It is interesting to note that antigens of this type, moreover, can be easily obtained from young cultures of the various bacteria in broth, and can be washed off the surfaces of bacteria like the pneumococcus, and influenza bacilli, making it more than likely that the sensitization is accompanied by a constant diffusing out from the focus of these materials. In comparing the production of these substances by living tubercle bacilli with extraction from dead tubercle bacilli, we found that when relatively large amounts of dead tubercle bacilli were infused in glycerin broth and, in parallel flasks, less than one-fifth of these amounts were planted on the broth in the living condition, the fluid surrounding the living tubercle bacilli was powerful in "tuberculin" substance as early as the third day when a slight amount only was present in the broth in which the dead tubercle bacilli had been infused.

Without going further into theoretical deductions from this, we are inclined to believe that reactions like the tuberculin, etc., reaction, are, indeed, phenomena in which antigen-antibody-like mechanisms are involved, but that here the process is probably transferred to the interior of the cell, and that because of the chemical and physical differences between the inciting substances involved, the manner of sensitization, both in time and quantity factors, is a different one.

The speed with which tuberculin sensitiveness develops in guinea pigs after infection with living organisms, that is, the 8th or 9th day, and the seriousness of the injury, inclines us to believe that such sensitization to these diffusible products may constitute an important factor in the injury of the body during bacterial infection.

Moreover, we have tried to simulate the conditions going on during infection in the body by injecting animals frequently with large amounts of dead tubercle bacillus material, and with Petroff have found that skin reactiveness can be developed if large amounts of dead tubercle bacilli are repeatedly administered.

This is particularly important in view of the fact that Krause has shown that skin hypersensitiveness and resistance to super-infection with tubercle bacilli, go hand in hand. Experiments on the artificial production of such increased resistance with dead tubercle products are going on.

While, therefore, this type of reaction typified by tuberculin, typhoidin, mallein and other bacterial skin reactions, cannot be properly spoken of as true protein anaphylaxis it, nevertheless, has many points in common with it, but also definite differences, the fundamental basis for which will probably in our opinion be found in the chemical and physical nature of the antigen involved.

The Anaphylatoxin Theory Applied to Bacterial Infection.—As one of the results of the attempts to explain anaphylactic phenomena by an intravascular reaction between antigen and antibody, a considerable amount of work was done with bacterial antigens, some of which took its departure from attempts to eliminate Pfeiffer's ideas of endotoxins.

Indeed the sudden liberation of endotoxins by immune sera had been regarded by Pfeiffer and others as the cause of the rapid death often ensuing in *immunized* guinea pigs when more than a definite maximum of cholera spirilla or other organisms was injected. In all these opinions the basic conception was that certain bacteria contained a characteristic preformed poison (endotoxin) upon the pharmacological properties of which the peculiar symptoms caused by each organism depended.

The earliest unambiguous statements of a conception differing from this original view of the nature of bacterial endotoxins, and approaching the later conceptions of Friedberger, are found, we believe, in the work of Vaughan.[37] In an article by him, published in 1908, Vaughan, after describing the incubation time occurring in man and animals after inoculation with typhoid bacilli, says: "The sickness begins when the animal body becomes sensitized and begins to split up the bacilli." By "splitting up" he means here, as in his other work [38] on protein split products, not a mere liberation of preformed poisons, but a chemical (enzymotic) proteolysis by which a poisonous group of the bacterial protein-molecule is set free.

The essential difference of this point of view from the endotoxin theory at first sight seems a trivial one—in the one case liberation of a preformed poison molecule, in the other liberation of a poison by the breaking up of a molecule. The difference, however, is a fundamental one. For, in the earlier theory, the specific element of the toxemia was in the nature of the different poisons—whereas in the view of Vaughan the lysin which breaks up the protein molecule is alone the specific element, the formed poisons being concerned as

[37] Vaughan. *Am. Jour. of Med. Sci.*, Sept., 1908.
[38] Vaughan. *Zeitschr. f. Immunitätsforsch.*, Vol. 1, 1909.

non-specific and alike, whether produced from colon bacilli, tubercle bacilli, or eggwhite.

Friedberger,[39] finally, in 1910, repeating with bacteria his experiments upon "anaphylatoxin" liberation from specific precipitates, succeeded in obtaining such poisons in the test tube by allowing fresh guinea pig complement to act upon sensitized bacteria.

These results were confirmed by extensive experiments carried out soon after this by Friedberger [40] himself with a number of collaborators.

The results of these investigations may be summarized as follows:

1. The action of alexin upon sensitized or unsensitized bacteria yields toxic substances which, injected into normal guinea pigs, produce the characteristic symptoms of anaphylaxis, with frequent death and typical autopsy findings.

2. These poisons ("anaphylatoxins") may be produced from any variety of bacteria, pathogenic and non-pathogenic.[41] (The organisms used in the earlier experiments were *Vibrio metchnikovi,* the bacillus of tuberculosis, the typhoid, prodigiosus, and subtilis bacillus, and *Aspergillus fumigatus.*)

3. The successful production of the poisons depends intimately upon the relative amounts of antigen (bacteria) and alexin used, and upon the time and temperature conditions under which the exposures are made.

4. The poisons can be produced from boiled as well as from native bacteria.

Although unsuccessful with none of the bacteria with which experiments were carried out, different species yielded the poison with varying degrees of intensity, and qualitatively the poisons were similar. *Bacillus prodigiosus,* though non-pathogenic, seemed to be one of the most favorable micro-organisms for such experiments.

The experiments of Friedberger and his associates were rapidly confirmed by Neufeld and Dold,[42] Kraus,[43] Ritz and Sachs,[44] and

[39] Friedberger. *Berl. klin. Woch.*, Nos. 32 and 42, 1910.

[40] Friedberger; Friedberger and Goldschmid; Friedberger and Szymanowski; Friedberger and Schütze; Friedberger and Nathan. *Zeitschr. f. Immunitätsforsch.,* Vol. 9, 1911.

[41] Neufeld and Dold, comparing virulent and avirulent strains of pneumococcus in this regard, have found that the virulence of the race has no relation to its yield of anaphylatoxin. Indeed the anaphylatoxins from various bacteria seem to be qualitatively entirely alike.

NOTE.—We wish to note here that we are giving Friedberger's theories and experiments at some length because they have had considerable influence on our conceptions of infection. We do not wish to create the impression that we accept them, however, and will discuss their fallacies later in the same chapter.

[42] Neufeld and Dold. *Berl. klin. Woch.*, No. 2, 24, 1911; *Arb. a. d. kais. Gesundheits Amt.,* Vol. 38, 1911.

[43] Kraus. *Zeitschr. f. Immunitätsforsch.,* Vol. 8, 1911.

[44] Ritz u. Sachs. *Berl. klin. Woch.*, No. 22, 1911.

many others,[45] and, though the conditions under which the anaphylatoxin formation took place were defined with slight variation by different workers, the essential features of Friedberger's claims were upheld.

It was further shown by Friedberger and Nathan that the conditions prevailing in the test-tube experiment in truth represent the processes taking place within the animal body. This they accomplished by injected bacterial emulsions into the peritoneal cavities of guinea pigs, killing the animals after several hours and examining the peritoneal exudates for their toxic properties. Centrifugalized, cleared of bacteria, and injected intravenously into other guinea pigs, these exudates produced the typical acute symptoms characteristic of the poisons obtained in test-tube experiments.

Friedberger now suggested that we may regard bacterial infection, after all, as the presence in the body of a living foreign protein —in this case varying in distribution and quantity by reason of the particular invasive properties of the given germ and the balance between these and the resistance of the host. It would not be necessary, therefore, to assume that the character of the disease is determined by the existence of different preformed "endotoxins." He believed that we may justly assume that the toxic substances appear only after protein cleavage of the bacterial bodies has been initiated by the action upon them of the serum components, and that the apparent specificity of the poisons, or differences between the toxemic manifestations of various diseases, may depend, not on differences in the pharmacological actions of these poisons, but rather upon variations in the invasive properties of the bacteria, both as concerns their quantitative distribution and their accumulation and localization in the infected body.

To support this assumption Friedberger pointed out the similarity in the clinical manifestations of several diseases in which the inciting bacteria are biologically very different, but in which the distribution and invasive properties are alike. For instance, lobar pneumonia caused by the pneumococcus is clinically very similar to that caused by the Friedlander bacillus, though the micro-organisms inciting them are extremely unlike each other. He drew a similar parallel between true cholera and cholera nostras, and we may add another striking example in the great similarity existing clinically between the various forms of acute and subacute septicemia in which a definite bacteriological diagnosis can rarely be made except by blood culture.

Conversely, the same micro-organism may call forth diseases which clinically, apart from the purely local manifestations, are very dissimilar, according to the localization and distribution of the bacteria.

[45] Izar. *Zeitschr. f. Immunitätsforsch,* Vol. 11, 1911.

These views of Friedberger constituted a serious objection to the "endotoxin" theory of Pfeiffer for the explanation of the toxic effects accompanying infection with organisms such as the typhoid bacillus, cholera spirillum, etc. However, the mechanism by which he explained the formation of the so-called "anaphylatoxins" was soon shown to be incorrect by investigations which indicated that the bacterial cell could not be justly regarded as the matrix of these poisons.[46]

The possibility that the bacterial cell might not furnish the actual chemical source of the poisons was first rendered questionable by the fact that Friedberger's anaphylatoxins could be produced as well from boiled as from living bacteria.

Such vague suspicion became a very definite doubt, in the light of the experiments of Keysser and Wassermann.[47] Keysser and Wassermann utilized the fact that certain serum elements may be absorbed out of serum if this is shaken up with indifferent suspensions such as barium sulphate or kaolin (aluminium orthosilicate).[48] They therefore substituted these insoluble substances for antigen, allowed them to absorb serum constituents, assumed by them to be amboceptor, out of normal and inactivated immune sera, and then allowed complement or alexin to act upon the "sensitized" kaolin.

In this way they obtained active and powerful anaphylatoxin, and claimed, in consequence, that the matrix of the poison was not in the bacterial antigen, but in the sensitizer or amboceptor, which was mechanically absorbed by the bacteria (as by the kaolin), and thus made amenable to the alexin action.

The experiments of Keysser and Wassermann found confirmation in the hands of other investigators, although the results of Neufeld and Dold, as well as our own, with this method were far more irregular than were those of Keysser and Wassermann. The many experiments on the production of anaphylatoxins (better Sero—or Proteo toxins) by placing serum in contact with pepton—with agar and a variety of other substances in fine suspension, followed. (See preceding chapter.)

Jobling and Petersen believe that, by the ordinary technique of anaphylatoxin production with bacteria and serum, most of the toxic substances originate from the serum proteins. The bacteria act merely by removing the antiferments from the serum, thereby setting free the ferments normally present in the serum, and permitting them to act upon the serum proteins. The result is cleavage and the production of toxic split products. This would explain such results

[46] See also Section on Anaphylatoxins in a preceding chapter.

[47] Keysser and Wassermann. *Folia Serologica,* Vol. 7, 1911; *Zeitschr. f. Hyg.,* Vol. 68, 1911.

[48] Kaolin emulsions will absorb amboceptor only out of diluted serum. Out of concentrated serum complement is completely absorbed. Friedberger u. Salecker, *Zeitschr. f. Immunitätsforsch.,* Vol. 11, 1911; Zinsser, from *Jour. Exp. Med.,* Vol. 18, 1913.

as those of Keysser and Wassermann. Jobling and Petersen supported their contention by experiments in which they have obtained typical anaphylatoxins by removing serum antiferments with chloroform, kaolin, and agar. They further showed that emulsions of bacteria actually do remove antiferments from fresh serum, and that the bacteria used in the process become more resistant to tryptic digestion in consequence.

Experiments of similar general import are those of Bronfenbrenner with the Abderhalden reaction.

It is thus clear that the interaction of bacteria and serum may produce toxic effects, certainly in the test tube, perhaps in the animal body. Whatever the mechanism of this process may be, it is not out of question that this phenomenon may have important bearing on the toxemia of infectious disease in which bacteria, as fine suspension, are in contact with plasma and tissues. This forces caution in further acceptance of the endotoxin theory—but the extent to which these processes are effective in the animal body is, as yet, quite unclear. We refer the reader to the preceding chapter in which anaphylatoxins are dealt with at considerable length.

Toxin Hypersusceptibility.—*Toxin hypersusceptibility,* which has occasionally been acquired by animals in the course of immunization with diphtheria and tetanus toxin, is usually classified with anaphylaxis, indeed is often cited as the earliest observation of this phenomenon. However, it is by no means clear that the two conditions are actually analogous, since in the case of the toxins we are dealing with antigens which are not only toxic in themselves, but against which neutralizing antibodies are formed in the reacting animal. This last fact alone would separate toxin hypersusceptibility sharply from true protein-anaphylaxis in that entirely different reacting-mechanisms seem to be called into play by the two varieties of antigen. It will be necessary, therefore, to discuss toxin hypersusceptibility at some length.

Probably the earliest authentically recorded observation is that of von Behring,[49] who determined, both for diphtheria and tetanus toxins, that animals once inoculated with these poisons were occasionally more sensitive to them subsequently than were normal animals. He spoke of "Gift Ueber empfindlichkeit" as a property acquired by reason of a preceding injection, and the observation was further developed by Knorr [50] in 1895, and by von Behring himself, in collaboration with Kitashima [51]—a few years later. These writers showed that guinea pigs which are treated repeatedly with small doses of diphtheria toxin may, under certain circumstances, not only fail to show immunity, but may even develop a susceptibility in-

[49] Von Behring. *Deutsche med. Woch.*, 1893.
[50] Knorr. Quoted from Otto, "Dissertation," Marburg, 1895.
[51] Von Behring u. Kitashima. *Berlin. klin. Woch.*, 1901.

creased to such an extent that doses far too small to injure a normal animal will cause their death. Again, in the case of diphtheria toxin similar observations were made upon horses by both Salomonsen and Madsen,[52] and by Kretz.[53] The last-named worker observed that horses that had been immunized with diphtheria toxin would often react to neutral mixtures of toxin and antitoxin by which normal horses were unaffected. This so-called "paradox phenomenon" was much discussed, and many theories advanced to explain it, a most ingenious adaptation of the side-chain theory being applied to it by Kretz [54] and by Wassermann.[55] They assumed that the partial immunization in such treated animals had in truth induced the formation of excessive receptors; that, in the stages of hypersusceptibility, however, these receptors had not yet been cast off from the cells. In consequence there was an excess of "sessile receptors"—by means of which the cell was rendered more exposed to toxin action than it was normally—it being still unprotected by the presence of freely circulating "antitoxin" receptors. The difficulties arising from the observation of similar hypersusceptibility in animals whose blood contained free antitoxin were disposed of by Wassermann by the convenient assumption of variations of affinity.

He assumed that the treatment with toxin, i. e., the intoxication, may induce a condition of higher affinity for the poison on the part of the sessile cell receptors, leading to a selective toxin-absorption by the cells and consequent greater susceptibility to injury. With Behring, he speaks of this as a "histogenic hypersusceptibility," implying an increased vulnerability of the tissue cells.

The analogy between these early observations and the phenomena which we now classify as anaphylaxis is unquestionably a striking one. However, it is doubtful, as Friedemann suggests, whether the two processes depend upon similar mechanisms. For, as we have seen in the case of the sensitiveness to toxin, we are dealing with primarily poisonous substances against which in the reacting animal neutralizing antibodies are found—a combination of conditions quite different from those with which we are confronted in hypersusceptibility against primarily harmless proteins. It is, of course, possible that the toxin hypersusceptibility is a true anaphylaxis against the toxin-protein—independent of the specifically poisonous nature of this substance. However, this is unlikely, since Löwi and Meyer have shown that with tetanus toxin, the symptoms of such hypersusceptibility are not those of anaphylaxis, but of increased but characteristic tetanus poisoning. Löwi and Meyer regard tetanus toxin

52 Salomonsen et Madsen. *Ann. de l'Inst. Past.*, 1897.
53 Kretz. Quoted from Otto in "Kolle u. Wassermann Handbuch," Ergängzungsband 2, p. 232.
54 Kretz. *Zeitschr. f. Heilkunde*, 1902.
55 Wassermann. "Kolle u. Wassermann Handbuch," Vol. 4, 479.

hypersusceptibility as a "summation"—meaning thereby that it depends upon an alteration of the cells of the spinal cord because of traces of the poison retained in them. When the toxin was given intraneurally no antitoxin formation occurred, but the animals developed a marked hypersusceptibility in the course of several weeks. This has been used as an argument that antibodies play no rôle in the process. However, the absence of circulating antitoxin would not seem to us to prove necessarily that no antitoxin was present in or on the cells. Similar reasoning has been found faulty in connection with protein anaphylaxis. The experiment, we believe, leaves us as uncertain as we were before.

A striking difference between toxin hypersusceptibility and protein anaphylaxis is the apparently uniform failure to produce toxin hypersensitiveness passively by the injection of antitoxic sera.

Thus we may summarize the conditions somewhat as follows: In conformity with the general laws of anaphylaxis, toxin hypersusceptibility is a process in which the inciting substance is an antigen, against which hypersusceptibility can be specifically induced by the successive injection of sub-lethal amounts. Differing from protein anaphylaxis, however, are the facts that the antigen in this case is an antitoxin producing toxic substance quite different from the sensitizer-producing ones represented by the proteins; that the symptoms elicited are not those of a general anaphylaxis, but rather the specific ones represented by the pharmacological action of the toxin, and that passive transfer with antitoxic serum has been unsuccessful.

It would be, therefore, merely a question of words to assign to these occurrences a definite place in the classification of phenomena of hypersusceptibility. We still believe that the most likely explanation is that the preliminary treatment gives rise to a certain degree of antitoxin production which remains sessile upon the cells and, in the animals in which the hypersusceptibility is noticed, thereby renders the tissues more susceptible to toxin injury. Passive sensitization may possibly have failed for the reason that it is very difficult to attain a balance in the passively injected animal in which the sessile antitoxic antibodies sufficiently outweigh the neutralizing ones in the circulation and we do know, from much work on antitoxin properties of circulating blood, and, conversely, from the experiments done, especially by Weil, on attempts to protect sensitized animals by the injection of antisera, that the union of toxin and antitoxin in the circulation takes place with much greater ease than does that of protein and sensitizer. Moreover, we know almost nothing about the absorption of antitoxin from the circulation by the body cells, and have no means at the present time of testing whether, after such absorption, if it occurs at all, the antitoxin is functionally intact, or whether it is destroyed. All these matters must be taken into con-

sideration when we deal with toxin hypersusceptibilities, and here again, as emphasized in our section on bacterial anaphylaxis, it is the nature of the antigen both chemically and physically, and the laws governing the union of the varieties of antigen with the antibodies which characterizes and varies the manifestations of various types of hypersusceptibility.

Toxic Action of Normal Sera.—There are a number of well-defined phenomena of acquired hypersusceptibility or sensitiveness which, in nature, seem closely analogous to true anaphylaxis as we understand it to-day, but regarding the mechanism of which the opinions of experimenters are still to some extent at variance.

Among the most important of these is the *toxic action of normal* sera when injected into animals of another species—a phenomenon which is now generally accepted as belonging in principle to the true anaphylactic phenomena, though this opinion is of comparatively recent formulation. The subject is of sufficient theoretical and practical importance to be considered in some detail.

The older studies of phenomena belonging to this category followed closely in the footsteps of experiments on transfusion, and as early as 1666 a commission of the London Royal Philosophical Society reported deaths following transfusion, alleging intravascular coagulation as the probable cause of death.

The cause of death following injections of foreign whole blood, blood cells, and serum has, since that time, occupied the attention of many workers whose studies need not be reviewed for our present purposes. Chief among them were Morgagni, Brown-Séquard, Magendie, and, more recently, Naunyn, Landois, and Ponfick.[56]

The work of Landois is of special interest in that he worked with blood serum free from cells, and attempted to correlate the occurrences after the injection of animals with the action of the serum upon the cellular blood elements *in vitro.* Landois observed both the solution of hemoglobin and hemagglutination, and was led to regard the action of serum upon erythrocytes as the primary cause of death after transfusion. His conception of the mechanism is apparently twofold. On the one hand, he believed that when small quantities of blood were transfused, a formation of fibrin (stroma-fibrin) was initiated in the stroma of the injured erythrocytes which led to coagulation and thrombosis in the capillaries of the central nervous system and lungs. In the case of the transfusion of rabbit's blood into dogs he attributed death to embolism in the pulmonary vessels due to "Massenhafte Verklebung der Kaninchenzellen im Hundeblut"—or, in other words, to hemagglutination.

Ponfick and others have disputed the validity of Landois' conclusions, but the basic principles of his explanations have been up-

[56] A brief historical review of this work can be found in the paper of Coca (1), *Virchow's Arch. f. path. Anat.*, 1909, Vol. 196, p. 92.

held within recent years by workers who have gone over the same ground with the aid of more modern methods. Two careful researches have appeared during the last two years in which the problem has been approached by different routes, but in which the general conclusions show much agreement. Coca,[57] investigating the cause of death following the injection of washed blood cells into animals of different species, concludes that in these cases death is due to mechanical obstruction of the pulmonary circulation owing to agglutination of the injected cells. It is important to note, however, that he adds in his conclusions the following paragraph: "The mere presence of specific agglutinins does not suffice, in the injection of 'toxic' erythrocytes, to occlude the pulmonary circulation. The coöperation of another factor must be assumed—a factor probably found in the capillary walls."

Loeb, Strickler, and Tuttle,[58] investigated the cause of death following the injection of normal dog and beef sera into rabbits. They correlated their animal experiments carefully with the action of the sera *in vitro* upon the blood elements of rabbits, and utilized the property of hirudin to inhibit the coagulation of blood, finding, in the case of dog serum, that injections of hirudin, while not always preventing death, at any rate prolonged life or necessitated an increase in the lethal dose. The conclusions of these authors are as follows: "Death following the injection of foreign serum is brought about by obstruction of the pulmonary circulation either by heaps of agglutinated erythrocytes or by fibrinous plugs. Dog serum and beef serum represent two different types. In the case of dog serum hemolysis of the blood cells of the recipient liberates substances attached to the stromata, which hasten coagulation. In consequence fibrin is formed which is carried into the pulmonary vessels and occludes them. In the case of beef serum death is due to hemagglutination."

The more recent understanding of the liberation of toxic bodies from blood cells by immune hemolytic sera, especially by the experiments of Friedemann cited above, have rendered it likely that a similar anaphylatoxin formation from the cells of the recipient may play a rôle in the toxic action of normal sera. And it is a fact, indeed, that such toxic sera are always hemolytic for the corpuscles of the susceptible animal.

An analysis of the toxic action of certain normal sera from this point of view has been made by Uhlenhuth and Haendel,[59] who, in studying the necrotizing action of beef serum injected into guinea pigs, attribute this action of the serum to a "complex process depending upon the coöperation of complement," but not identical with the hemolytic mechanism. The toxic action of such serum, how-

[57] Coca. *Virchow's Archiv,* Vol. 196, 1909.
[58] Loeb, Strickler, and Tuttle. *Virchow's Archiv,* Vol. 201, 1910.
[59] Uhlenhuth and Haendel. *Zeitschr. f. Immunitätsforsch.,* Vol. 7, 1910.

ever, they separate from the necrotizing action, concluding that this is independent of complement, and more thermostable than either the mechanism causing necrosis or that responsible for hemolysis.

Studies of the writer [60] on the toxic action of goat serum for rabbits have shown that, contrary to Loeb, Strickler, and Tuttle, hemagglutination and blood coagulation can be excluded as causes of death in some instances and that, in agreement with Uhlenhuth and Haendel, the toxic action may be due to action of the serum not necessarily identical with the hemolysins. Unlike Uhlenhuth and Haendel, however, it seemed clear that the participation of alexin was definitely necessary—the process being probably entirely analogous to Friedemann's results with immune hemolytic (cytolytic) sera. The poisonous action of dissolved hemoglobin could be excluded.

Thus, it would seem that foreign serum can be injurious to any given species of animals in a number of ways. Before any other mechanism is suggested, it would seem of the greatest importance always to exclude thrombosis by hemagglutination. In addition to this, however, processes of hemolysis with the formation of toxic substances must be considered as possibilities. From our own work we would further assume that a serum which has hemolytic properties for the red blood cells of another species is, at the same time and for the same reasons, probably capable of injuring the tissue cells of the injected animal by processes not visually observable, for our experiments have led us to believe that if normal hemolytic substances are present in such a serum, similar, perhaps antibody-like substances, for the remaining components of the cell protein may also be present. This is hard to prove, but is suggested by the work reported above, and would appear to us a logical possibility. Finally, it must not be forgotten that in such complex processes, alterations of the coagulation mechanism of the injected animal are not at all unlikely, and, as we have seen in the discussion of the anaphylatoxin theories, toxic action is also associated with processes which initiate coagulation.

An Attempt at the Coördination of Various Phenomena of Hypersusceptibility.—In concluding the section of this book dealing with the phenomena of hypersusceptibility, we cannot resist the temptation of attempting briefly to present our views as to the possible coördination of the various conditions. In protein anaphylaxis, itself, we have a condition in which the animal body becomes delicately susceptible to non-diffusible protein antigens to which specific antibody production occurs whenever the inciting substance penetrates the physiological interior of the body. We have suggested that the physical inability of these substances to penetrate into the cell may possibly have causal relationship to their antigenic func-

[60] Zinsser. *Jour. Exp. Med.*, Vol. 14, 1911.

tions, the production of antibodies representing the mechanism by which the body cells eventually react with the non-diffusible proteins. This conception thus suggests a relationship between antigenic properties, non-diffusibility and molecular size. The antigen-antibody mechanism, it can no longer be doubted, lies at the bottom of the mechanism of protein anaphylaxis, and that the reaction which causes injury is predominently localized upon the cellular elements of the body, is also beyond question.

In *serum sickness,* we have a condition in which the antigenic nature of the inciting agent, the probable relation with antibody production, the coincidence of local and systemic symptoms, form such a striking analogy to anaphylaxis in lower animals that nothing but the most decisive reasons could persuade us that it is an unrelated process. Indeed, the occasional rapid appearance of the reaction on first injection, finds explanations which have at least as much basis as the complete throwing out of all the other analogies because of this one discrepancy; and the appearance of serum sickness without antibodies in the circulation is, as we have also seen, easy to explain. (Vide infra.) Moreover desensitization and parallelism with typical skin hypersensitiveness, further strengthen the analogy of serum sickness with protein anaphylaxis.

In the *food idiosyncrasies* we have another set of conditions in which the inciting substance is antigenic, and in which a considerable proportion of the cases can be very definitely explained by sensitization, inasmuch as sensitization through the intestinal canal has been proved to be possible in guinea pigs. The peculiarities of this condition, we think will find a clearer comprehension, when we take more seriously into consideration the possibility of a localization of the sensitizing process in the intestine, due to a more prolonged contact of the antigen with the cells at the point of entrance. These cells are perhaps thereby much more highly sensitized than the body as a whole when antigen is injected intravenously, and reaches all the cells in a diluted condition. This is merely speculation; but views that tend to separate this condition from protein anaphylaxis are also very largely based on speculative reasoning from complex experimental results, and a trellis of speculation seems to us very useful in this subject, if not too dogmatically presented. In food idiosyncrasies, again, desensitization and a gradual return after this to the sensitive condition, link the process with anaphylaxis. Our views of the hereditary influence of this condition have been stated in a previous section. (See section on food idiosyncrasies.)

If we look upon *hay fever* in the same way, practically all the remarks we have made about food idiosyncrasies are pertinent. Here the localized sensitization, both as to point of sensitization and as to development of symptoms, indicates a powerful influence of the localization of the process, and in other respects, such as the heredi-

tary influence, skin reactions, and desensitizations, analogy is established. Here we are dealing, however, with a substance, the antigenic nature of which has been questioned. While the responsible substances here may in the end be shown to be non-antigenic, we must regard this still as undecided, in view of the experiments of Parker in our own laboratory, in which it was proven that an antigenic material could be extracted from pollen, investigations which have been entirely neglected in the discussion of the subject by other writers.

In *bacterial hypersensitiveness,* we see two processes, a true anaphylaxis to the protein constituents of the bacteria, and another one, also specific, such as tuberculin, typhoidin, etc., reactions, which differ in some of their manifestations from true anaphylaxis, especially in the more destructive and intracellular nature of the injury produced in the sensitized animals, and from the fact that infection with a constant feeding into the animal of certain bacterial products, seems to be a prerequisite for the acquisition of the sensitiveness. While this is probably not true in the extreme, yet in general spontaneous occurrence it is the case. Here as we have shown, ourselves, the inciting substance involved is a material which, if it finally turns out to be a true protein, nevertheless differs in many important respects from the ordinary proteins. It is obtained by extraction of the bacteria, and left as a residue after boiling with acid, even in some cases after autoclaving in an acid state, in an attempt to remove all proteins, and we believe that these substances of which the tuberculin extracts studied by us are an example, may perhaps represent the "Haptenes" foretold by Landsteiner. They react with antibodies, but, so far, have not incited them. With such fundamental differences from ordinary proteins in the inciting substances, manifestations and laws governing sensitization may well differ from those governing protein hypersensitiveness, and still be based upon a reaction of these substances with a cellularly placed antibody. It may be, though we have not yet been able to prove it, that the intracellular nature of the injury in these cases, as evidenced in tuberculin reactions in contrast with the ordinary urticarial protein cutaneous reactions, is related in some way to the diffusibility of the materials in question. This, again, however, is largely speculative.

In the *drug idiosyncrasies,* we have a group of reactions in which the inciting substance is clearly not antigenic in itself. There are two possibilities here; either that these substances have close analogy to protein anaphylaxis in that the drug in question undergoes a definite chemical reaction with the blood and tissue proteins, producing an altered antigen, such as the methylated and azotized antigens of Pick, Landsteiner and others, and then act just like an antigen, subject, perhaps, to laws varying in accordance with the intracorporal formation of the antigen; or the whole process may take place intra-

cellularly, in that the drug diffuses into the cells, there entering into combinations which then become antigenic. This would be to some extent indicated by the apparent impossibility, hitherto, of passively transfering any of the drug hypersensitivenesses. However, we admit that the drug idiosyncrasies are the most vague, so far, and the most distant from true protein anaphylaxis in analogy. Nevertheless, the fundamental analogy remains, namely, that individuals may become either hypersusceptible or resistant, that is, relatively immune to the primary injurious action of the drugs; and it is important to note that when hypersusceptibility occurs, this hypersusceptibility is not evidenced by increased physiological action of the drug, but by a general type of reaction which for the species of animal, man, shows great similarity for a variety of drugs absolutely independent of the physiological action of the drug itself.

Thus, while we are faced with a large variety of hypersensitive states and while classifications, such as those of Doerr and Coca are of considerable preliminary value, it would be a pity, we believe, if the erection of such fences of division at the present time should influence investigators to overlook fundamental similarities.

CHAPTER XX

THERAPEUTIC IMMUNIZATION

(Passive Immunization and Serum Treatment)

Therapeutic Use of Diphtheria Antitoxin

It is not consistent with the purpose of this brief treatise to discuss extensively the therapeutic benefits obtained by serum therapy in diphtheria. We can convey briefly an adequate idea of this by citing some of the tables given by Northrup in Nothnagel's "Encyclopedia of Practical Medicine," American Edition, Volume on Diphtheria, etc., p. 143. These figures are taken from the statistics of the New York Board of Health, which began treatment of diphtheria with antitoxin in January, 1895. Dr. Northrup states, however, that serum treatment cannot be considered to have been in general use until some time later.

Without Antitoxin

Year	Cases reported	Deaths	Mortality, per cent.
1891	5,364	1,970	36.7
1892	5,184	2,106	40.0
1893	7,021	2,558	36.4
1894	9,641	2,870	29.7
Total	27,210	9,504	Avg. 34.9

With Antitoxin

Year	Cases reported	Deaths	Mortality, per cent.
1895	10,353	1,976	19.0
1896	11,399	1,763	15.5
1897	10,896	1,590	14.5
1898	7,173	919	12.8
1899	8,240	1,085	13.1
1900	8,364	1,176	14.0
Total	56,425	8,509	Avg. 15.0

Table taken directly from Northrup, *loc. cit.*

From this table there appears a reduction of 58 per cent. in mortality and a similar drop is evident from the German statistics of Dieudonné,[1] from those of Welch, and many others.

[1] Dieudonné. *Arb. a. d. kais. Gesund.*, Vol. 13, 1897.

It should be considered, moreover, in reading such statistics that they are made on gross mortality reports without elimination of the many cases that have not come under observation until too severely diseased to react to any form of treatment. The reason for the failure to obtain results with antitoxin when the cases have proceeded beyond a certain stage of intoxication will become evident when we consider the manner of absorption of the poison in a succeeding paragraph. The mortality sinks to between 8 and 9 per cent., when such cases are omitted, as is shown by the collective investigations of the American Pediatric Society in 1896—figures which we take also from Northrup's comprehensive study. This purely statistical evidence, however good, is further reënforced by the unquestionable and considerable diminution of emergency operations,[2] such as intubation and tracheotomy, since introduction of the antitoxin. Moreover, there is the manifold clinical evidence of benefit, after the serum treatment, familiar to every practicing physician.

Although the injection of antitoxin is of benefit by whatever route and in whatever quantity it may be given, nevertheless recent experimental investigations have taught us much regarding the proper use of this therapeutic agent. Especially interesting are the investigations of Meyer,[3] who showed the extreme importance of an early use of the antitoxin. Apparently, as we have mentioned in another place, like tetanus antitoxin, the diphtheria poison may be in part absorbed directly by the nerves.[4]

There is apparently a great difference in therapeutic efficiency, according to the method by which the serum is administered, a difference probably depending upon speed of absorption. Berghaus[5] showed that intravenous injection is 500 times more potent therapeutically than the subcutaneous, and 80 to 90 times more so than the intraperitoneal injection. Schick, for this reason, in discussing this problem from the clinical point of view, lays special stress upon the speed of administration. He says: "Not only days but hours are of great importance." He bases this opinion largely upon the fact that the toxin which has already united with the nerve substance can no longer be neutralized by antitoxin injections—perhaps owing to its union with constituents of the cells.

According to the experiments of Meyer and Ranson diphtheritic paralysis may follow even when vigorous serum treatment has been employed. For, according to them, only the toxin which has reached the central nervous system through the circulation can be influenced

[2] Siegert. "Jahrbuch f. Kinderheilkunde," Vol. 52, cited after Wernicke.

[3] Meyer. *Berl. klin. Woch.*, Nos. 25, 26, 1909; *Arch. f. exp. Path. u. Ther.*, Vol. 60, 1909, and *Berl. klin. Woch.*, No. 45, 1911.

[4] For a thorough discussion of these conditions see Schick, *Centralbl. f. Bakt.*, Rev. Vol. 57, 1913, "Report of 7th Meeting of the Mikrobiol. Gesell.," Berlin, 1913.

[5] Berghaus. Cited from Schick, *loc. cit.*

by the serum, but no effect is possible upon the fraction which has been absorbed from the nerve endings directly.

Schick,[6] on the basis of extensive experiments, comes to the conclusion that the subcutaneous injection of 1,000 to 2,000 units in diphtheritic cases has an immunizing value which protects the tissues from further injury and leads to cure only if, at the time of injection, the lethal dose has not yet united with the sensitive cells. "If," he states, "we wish to obtain antitoxic action upon toxin which has already gone into action before the injection of the serum, then results can be obtained both in man and in animals only if a great deal of antitoxin is injected intramuscularly or intravenously." [7]

Interesting also from a clinical point of view are the studies of Schick,[8] Hahn,[9] and others [10] upon the presence of antitoxin in the blood of normal, untreated individuals at different ages. These investigations were carried out by the intracutaneous method of toxin and antitoxin determination described in greater detail in a later section. The following table, taken from the article of Hahn, illustrates the experience, in such investigations, both of Schick and of Hahn himself. The determinations were carried out upon individuals who had never had diphtheria, as far as could be learned.

Age		Cases with antitoxin serum	Cases without antitoxin serum	Highest antitoxin value in 1 c. c.*
Schick	Newborn	11	0	under 1.5 units
	0–1 year	1	3	0.11 unit
Hahn	2–10 years	7	5	1.0 unit
	11–20 years	8	9	0.75 unit
	21–30 years	9	5	2.5 units
	31–40 years	5	1	0.25 unit
	41–65 years	2	8	2.5 units

* Table taken directly from Hahn, *loc. cit.*

The table shows that in newborn children there is almost regularly a definite and sufficient protective value in the serum which diminishes up to the first year, so that at the end of the first year three out of four individuals have no antitoxin in their serum. In subsequent years up to the age of 40 an increasing percentage of people have sufficient amounts of diphtheria antitoxin in their blood. After the age of 40 an increasing percentage is without such protection. The first observation, that newborn children usually possess considerable amounts of antitoxin, is very probably due to passive

[6] Schick. *Loc cit.*
[7] Schick. *Loc. cit.*, p. 32.
[8] Schick. "Uber Diphtherimmunität," Wiesbaden, 1910.
[9] Hahn. *Deutsche med. Woch.*, Vol. 38, 1912, No. 29, p. 1366.
[10] Karasawa and Schick. *Zeitschr. f. Kinderkrankheiten*, 1910, and "Jahrbuch. f. Kinderheilkunde," 1910.

immunization by the blood of the mother, a fact which we have mentioned in another place. The original method by which such measurements can be made is described in a subsequent section on the intracutaneous method of determining toxin and antitoxin.

Another convenient method of titrating antitoxin in human serum, colostrum, milk, etc., has been described by Kellogg.[11] This method has been used with much convenience and accuracy in our laboratory by Kuttner and Ratner. The preliminary measurements on which Kellogg's method is based are the following: In guinea pigs 1/300 of a M L D gives a definite intracutaneous reaction which is at its height in about 48 hours; 1/40 of a M L D is the smallest amount of toxin that will produce necrosis. Since the L + dose of toxin is the amount that will neutralize one standard unit of antitoxin, leaving one minimal lethal dose of toxin free, it follows that if we make a series of mixtures in which the fraction of the L + dose is accurate, known and constant with varying amounts of patient's serum, milk, colostrum or other materials and with such mixtures carry out skin reactions on guinea pigs we can determine, with fair accuracy, the antitoxic strength of the specimen in units of antitoxin.

Kellogg makes a mixture of toxin in which the amount contained in 1 c. c. equals 1/30 the L + dose. Now, if 1 c. c. of such a toxin mixture is mixed with 1 c. c. of serum containing 1/30 unit of antitoxin, this will leave a balance of 1/30 minimal lethal dose, and mixtures of 0.1 c. c. of each will have a surplus of 1/300 minimal lethal dose of toxin. The volume of mixture injected is 0.2 c. c., which, therefore, represents 1/300 of the L + dose of antitoxin, plus whatever antitoxin may be contained in 0.1 c. c. of the serum.

Readings can be made from 48 to 72 hours. If the serum contains exactly 1/30 a unit of antitoxin per c. c., which amount, it will be remembered, is the arbitrarily determined one that is supposed to still give protection in human beings, 1/300 minimal lethal dose of toxin will remain free, and will still give the mild reaction determined for this.

If the amount of antitoxin is greater than this, no reaction at all will appear. If the amount of antitoxin is less than this, necrosis will be evident. By titrating in one direction or another, a fairly accurate idea of the antitoxin content of the serum can be obtained. Appropriate controls are, of course, always made.

The work of Schick, that of J. Henderson Smith, and recent studies by Park and Biggs promise to alter considerably the methods of antitoxin therapy as at present in use in diphtheria. Smith measured the speed of absorption of antitoxin injected subcutaneously into the abdominal wall of a healthy man. His results are shown in the following table, which we take from his communication (page 213):

[11] Kellogg. *Jour. A. M. A.*, June 10, 1922. Kellogg's method is really a convenient modification of the method of Römer which is described on p. 536.

TABLE V

One c. c. of the patient's serum contained:

Before injection	No demonstrable antitoxin
5 hours after injection	0.1 unit antitoxin
14 hours after injection	0.225 unit antitoxin
32 hours after injection	0.68 unit antitoxin
44 hours after injection	1.0 unit antitoxin
3 days after injection	1.3 units antitoxin
4 days after injection	1.3 units antitoxin
6 days after injection	0.68 unit antitoxin
13 days after injection	0.17 unit antitoxin
15 days after injection	0.14 unit antitoxin
20 days after injection	0.08 unit antitoxin
27 days after injection	No demonstrable antitoxin *

* J. Henderson Smith. *Jour. Hyg.*, Vol. 7, 1907, p. 205.

Park and Biggs [12] have made similar studies and have contrasted the speed of absorption after subcutaneous administration with that after intravenous injection, basing their curves upon careful measurements of the sera of the treated patients. We reproduce their charts as given in their recent publication.

It is apparent from these charts, as well as from the work of Henderson Smith, that antitoxin, subcutaneously given, is slowly absorbed, and does not reach its maximum concentration in the blood stream until forty-eight hours or more after the injection. It follows that, as Park and Biggs point out, it is more rational to inject a single adequate dose than to divide the dosage and inject at intervals. They have obtained results in animal experiment which graphically illustrate this principle. A rabbit which had received ten fatal doses of toxin intravenously was given a total of 50 antitoxin units in divided doses as follows: 100 units after twenty minutes, 100 after 40 minutes, and 150 units each after 60 and 80 minutes. This rabbit died. Another animal given the same dose of toxin received 200 units of antitoxin twenty minutes later and lived. The amount necessary to save life in rabbits receiving ten fatal doses intravenously was as follows:

Given after 10 minutes	5 units antitoxin
Given after 20 minutes	200 units antitoxin
Given after 30 minutes	2,000 units antitoxin
Given after 45 minutes	4,000 units antitoxin
Given after 60 minutes	5,000 units antitoxin
Given after 90 minutes	No amount

These extremely important experiments of Park and Biggs bear out the opinion of Schick and show beyond question that the proper way to give antitoxin is to give a single adequate dose as early as

[12] Park and Biggs. Collected Studies from the N. Y. Department of Health, Bureau of Laboratories, Vol. 7 1912-1913, p. 27.

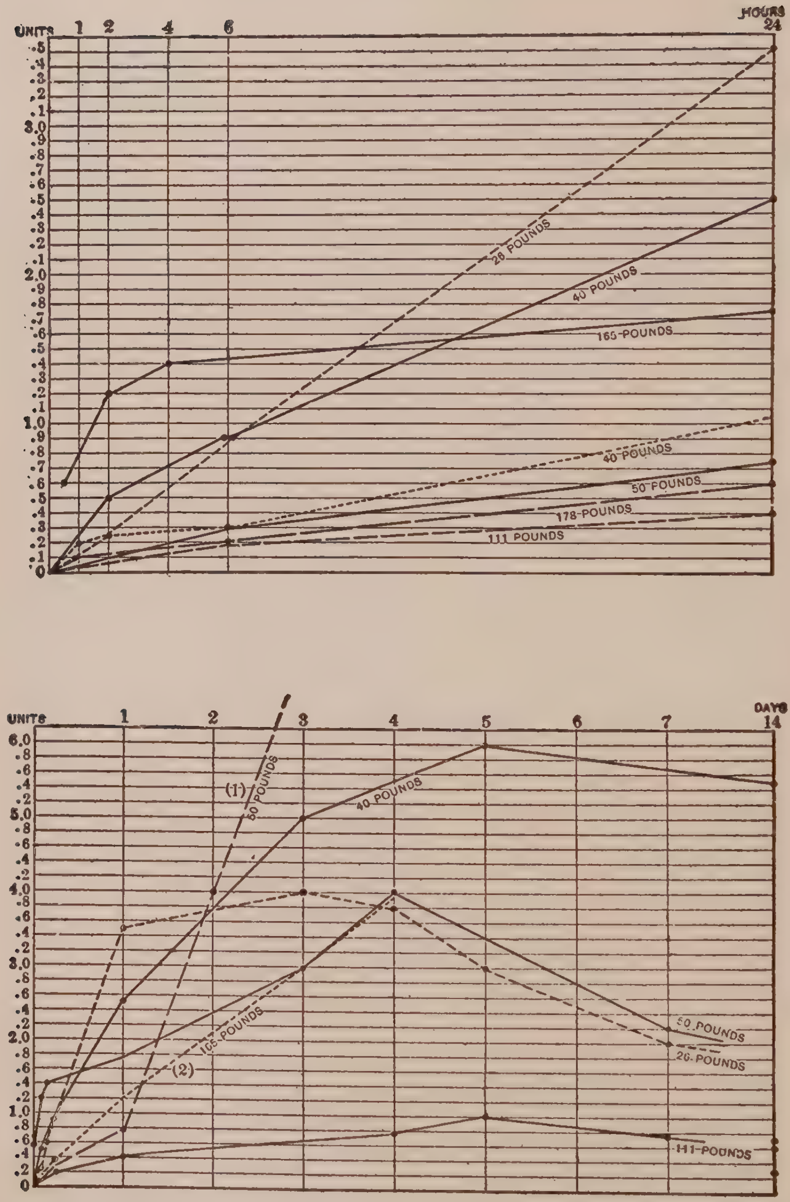


CHART I.—Showing the extent and rapidity of absorption of 10,000 units of antitoxin given subcutaneously. Each line represents the antitoxin content of 1 c. c. of blood at different intervals of time. (From Park and Biggs, *loc. cit.*)

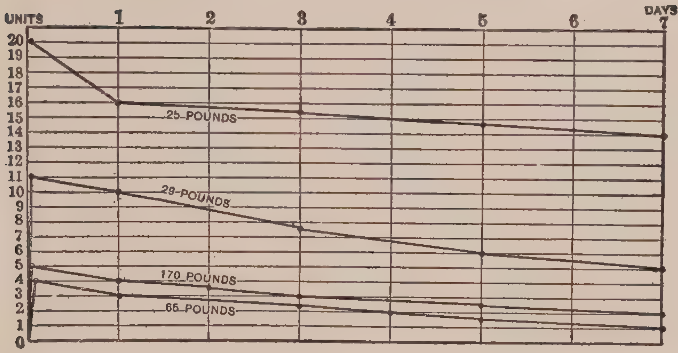


CHART II.—The antitoxic power of human blood after an intravenous injection of 10,000 antitoxic units. (From Park and Biggs, *loc. cit.*)

possible. They emphasize the fact that probably the most important single point in the specific therapy of diphtheria is the speed with which the diagnosis can be made and the antitoxin given. At the Department of Health the dosage now employed, as given by Park and Biggs, is the following:

	Units in Cases			
	Mild	Moderate	Severe	Very Severe
Infants under 1 year..........	2,000	3,000	10,000	10,000
Children 1 to 5 years.........	3,000	5,000	10,000	10,000
Children 5 to 9 years.........	4,000	5,000	10,000	15,000
Persons over 10 years.........	5,000	10,000	10,000	20,000

Practical Considerations Connected with Diphtheria Antitoxin Production and Standardization

The conditions which govern the active production of toxins by bacteria in culture media are not only of great theoretical interest but possess unusual practical value in that the most important factor for the successful production of a strong antitoxin consists in the preliminary preparation of a potent toxin. The bacterial true toxins are all "exotoxins" in that they are soluble, moderately diffusible substances which pass readily from the bacterial bodies to the environment, and for this reason can be obtained most readily by the cultivation of the bacteria upon fluid media and subsequent filtration of the cultures through earth or porcelain filters.

The choice of culture or strain is an important element in this procedure, since within the same species of toxin-producing micro-

organisms there is much variation in the speed and energy of toxin production. Thus for unknown reasons some strains of diphtheria bacilli will far outstrip others in this respect. An excellent illustration of this is the experience of Park and Williams [13] with two diphtheria cultures—a very virulent and a very weak one. Of the former, 0.002 c. c. of a forty-hour bouillon culture killed a guinea pig, while of the latter 0.1 c. c. of a similar culture was necessary for the same result.

In the case of tetanus, cultural differences do not seem to be as common. Individual strains also may gain or lose in toxin-producing powers, according to the method of handling them which is practiced. It is stated,[14] for instance, that a diphtheria culture will lose in energy of toxin production if permitted to grow without sufficiently frequent transplantation. However, transplanted on solid media with reasonable frequency, these bacteria show a remarkably constant toxin production. A well-known strain, the Park-Williams No. 8, now in use in many antitoxin laboratories throughout the world, has persisted for over 15 years in producing a strong toxin. There are occasional strains among toxin-forming species which are entirely devoid of this property. Diphtheria bacilli which were virulent while possessing all the other cultural characteristics of the group have been described, but appear, from the experience of the writer, to be rather uncommon.[15] Of tetanus bacilli little is known in this respect.

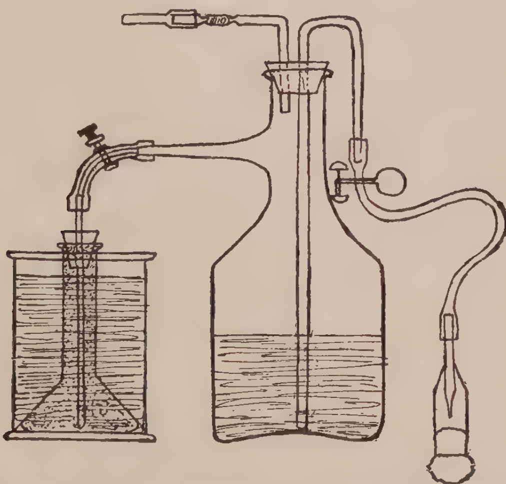
Given a powerfully toxic strain of the proper bacteria the method of cultivation is also of great importance in influencing the eventual yield of poison. These relations have naturally been studied with the greatest care in the case of diphtheria and tetanus bacilli, since in these cases there has been the greatest practical application for such knowledge.

In the case of diphtheria, though toxin will be produced on all media on which the bacillus grows easily, the most favorable medium for this purpose is a slightly alkaline broth made of lean beef or veal infusion and containing peptone. Since acid formation hinders the production of toxin, Martin [16] has suggested fermentation of the muscle sugar with yeast, while Theobald Smith [17] recommends preliminary fermentation with *Bacillus coli.*

Park and Williams [18] regard this as unnecessary. They recommend a 2 per cent. peptone broth made of veal. This is neutralized to litmus and 7 to 9 c. c. of normal NaOH solution to the liter are

[13] Park and Williams. "Pathogen. Micro-organ.," New York, 1910.
[14] Park and Williams. *Loc. cit.*
[15] Zinsser. *Jour. Med. Res.*, N. S., Vol. 12, 1907.
[16] Martin. *Ann. de l'Inst. Past.*, 1896.
[17] Th. Smith. *Jour. Exp. Med.*, Vol. 4, 1899, p. 373.
[18] Park and Williams. *Jour. Exp. Med.*, Vol. 1, 1896.

added. In such a medium at 37.5° C. the production of toxin begins within 24 hours and reaches its highest point in from five to ten days. When at its height the process must be stopped and the cultures exposed to a lower temperature, otherwise rapid deterioration takes place because of the instability of the toxin. Even when kept cold and in the dark this deterioration proceeds steadily though slowly. At first, however, even under these conditions a comparatively extensive loss of toxin goes on—a process sometimes spoken of as "maturing of the toxin"—after which the poison strikes a fairly constant and very gradual rate of weakening, and is, comparatively speaking, stable.



Apparatus Arranged for the Sterile Filtration of Diphtheria Cultures in Toxin Production.
(After Rosenau, *U. S. Hyg. Lab. Bull.* 21, 1905, p. 38.)

In the United States Hygienic Laboratory in Washington, according to Rosenau, the recommendations of Theobald Smith are largely followed in the production of toxin. The procedure is as follows:

The culture medium, "Smith's Bouillon," is prepared from chopped beef from which fat and tendon have been cut out. This is adjusted by plenolphthalein titration to 0.5 per cent. acidity. It is then placed into Fernbach flasks and inoculated on the surface with a Park-Williams bacillus No. 8. The flasks are incubated for 7 days at 37.5° C. The reaction of the medium after such incubation is determined, and flasks showing an acidity of 1.5 or over are discarded. The usual reaction at the end of incubation is 0.6 to 0.8 per cent. acidity. This broth is filtered through Berkefeld filters or porcelain candles.

Toxin so prepared is now tested and its L_0 and L_+ doses determined by the methods described above. Rosenau[19] states that poisons are discarded as containing too large a proportion of toxon if the difference between L_0 and L_+ is greater than 15 M L D. The toxin is now set aside in flasks for the process which Rosenau calls "seasoning." At intervals of about a month it is retested and finally it is found that the rate of toxoid formation decreases and the poison reaches a period of equilibrium. It can now be used for accurate determination of the L_+ dose, and this is done from careful measurements on a large number of guinea pigs.

Examples[20] of such measurements, abbreviated for the sake of simplicity, are given in the following tables:

Toxin Determinations of M L D or "T"

Dose in c. c.		Result
0.03	=	death in 1½ days
0.02	=	death in 1½ days
0.01	=	death in 2 days
0.008	=	death in 3 days
0.006	=	death in 3½ days
0.005	**=**	**death in 4 days M L D**
0.004	=	death in 6 days
0.003	=	death in 8 days
0.002	=	late paralysis
0.001	=	well in 16 days.

Toxin Determination of L_+ Dose

1 Antitoxin unit + 0.2 c. c.	=	0
1 Antitoxin unit + 0.21 c. c.	=	0 = L_0
1 Antitoxin unit + 0.22 c. c.	=	local infiltration
1 Antitoxin unit + 0.23 c. c.	=	fatal in 17 days
1 Antitoxin unit + 0.24 c. c.	=	fatal in 14 days
1 Antitoxin unit + 0.26 c. c.	=	fatal in 9 days
1 Antitoxin unit + 0.28 c. c.	=	fatal in 6 days
1 Antitoxin unit + 0.29 c. c.	**=**	**fatal in 4 days = L_+**
1 Antitoxin unit + 0.3 c. c.	=	fatal in 3 days

The production of antitoxin is carried out by the graded injection of antitoxin into horses. Young, healthy horses are chosen, tested for freedom from glanders, and the first injections are made either with toxin attenuated by the addition of Lugol's solution or terchlorid of iodin, or, as in the New York Health Department, the first injections consist of mixtures of toxin and antitoxin. We take our description largely from the account given by Park.[21] The first injection consists of 12 c. c. of toxin (M L D 1/400 c. c.), together with 100 units of antitoxin. After the reaction from such an injection has completely subsided—after 3 to 5 days—a second injection

[19] Rosenau. *Hyg. Lab. Bull.* No. 21, April, 1905.

[20] Examples are taken from measurements reported by Rosenau, *loc. cit.*

[21] Park and Williams. "Pathogenic Bacteria," p. 213.

is given of toxin without antitoxin; then 15 c. c., 45 c. c., 55 c. c., 65 c. c., 80 c. c., 95 c. c., 115 c. c., 140 c. c., etc., the intervals between injections being about three days and depending upon the reaction of the horse and the speed with which it entirely recovers from the preceding injection. In a particular case cited by Park 675 c. c. of toxin could be given by the 60th day; in this case by the 28th day the horse was yielding 225 units to the c. c.; on the 40th day, 850 units; on the 60th day, 1,000 units.

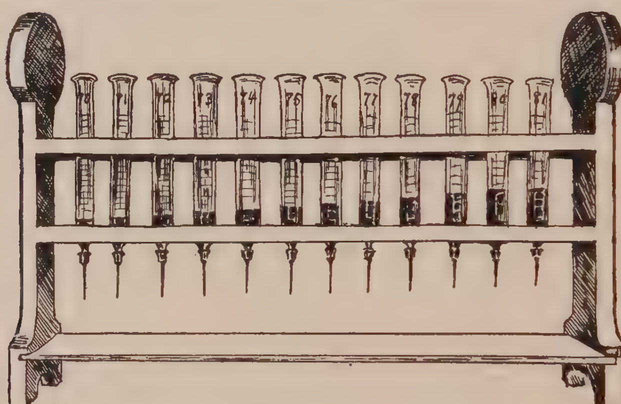
The determination of the antitoxin unit, carried out from time to time on the serum of such a horse against the L_+ dose described in our preceding table, would be carried out as follows:

In all such standardization great care must be taken in employing accurately standardized glassware. Rosenau recommends employing "capacity instruments" rather than "outflow instruments." Dilutions of unknown antitoxin are made in 0.85 per cent. sterile salt solution. As a basic dilution one part of the antitoxic serum to nine of the salt solution gives 1/10 c. c. to each cubic centimeter, and from this initial dilution further dilutions may be easily made as follows: 1 c. c. of dilution I. + 9 c. c. salt solution = 1-100, etc. A series of mixtures is then made in each of which the quantity of toxin equals the L_+ dose, and in which the quantity of antitoxin varies within a wide margin of the limits of strength to be expected. This is illustrated in the following table:

L_+ (0.29 c. c.) + 1/500	c. c. of antitoxic serum	= lives
L_+ (0.29 c. c.) + 1/600	c. c. of antitoxic serum	= lives
L_+ (0.29 c. c.) + 1/700	c. c. of antitoxic serum	= lives
L_+ (0.29 c. c.) + 1/800	c. c. of antitoxic serum	= dies in 8 days
L_+ (0.29 c. c.) + 1/900	c. c. of antitoxic serum	= dies in 4 days
L_+ (0.29 c. c.) + 1/1,000	c. c. of antitoxic serum	= dies in 2 days

In the above tables, according to our previous definition of the antitoxin unit, the serum would contain 900 units to the cubic centimeter, since 1/900 c. c., injected together with the L_+ dose of the standard toxin, resulted in the death of the guinea pig in four days. In order to allow a margin of safety Rosenau and others have suggested that the unit should be determined, not by the quantity of antitoxin, which delays death by the L_+ dose for four days, but rather by the quantity which, with the L_+ dose, results in saving the life of the guinea pig. According to this latter standard the serum employed in the table would be spoken of as containing 700 units to the cubic centimeter. Of course the tabulated measurements are rough, leaving an undetermined zone of 100 units. The exact number of units to the cubic centimeter could, of course, be determined with greater accuracy by now carrying out another series of tests in which the amount of serum varied between 1/700 and 1/900 of a cubic centimeter.

In carrying out such a standardization the toxin is diluted so that the L_+ dose is contained in 2 c. c. This can easily be done. For instance, in the above the L_+ dose being 0.29, it merely necessitates adding to each 0.29 c. c. of toxin 1.71 c. c. of salt solution, to each 2.9 c. c., 17.1 c. c. The antitoxin also is made up in such a way that the required dilution is contained in two cubic centimeters, since a total volume of 4 c. c. has been agreed upon as standard for these tests, the injected volume having much influence upon the speed of absorption. In using the so-called Rosenau syringe, shown in the figure for the standardization of antitoxin, the antitoxin is made up to 1 c. c. in each case, so that 1 c. c. of salt solution may be added to wash out the syringe after injection of the mixture. The mixtures



Battery of Rosenau Syringes Prepared for Antitoxin Standardization. (Taken from Rosenau, *U. S. Hygienic Bulletin* 21, 1905.)

can be made directly in these syringes or in test tubes, and are allowed to stand one hour at room temperature, so that there may be time for complete union. If the mixtures are made directly in the syringes the needles are dipped into sterile vaselin, which closes them and prevents leakage while standing. The mixture is then forced out of the syringe with a rubber bulb, thus ensuring complete injection of all the fluid. As Rosenau states, much depends on the guinea pigs. They must be of standard weight, about 250 grammes, well fed and cared for, and must not be descendants of pigs that have shown marked or unusual resistance to diphtheria toxin. This, as Theobald Smith has shown, occasionally happens.

The antitoxic serum as obtained from the horse directly may be concentrated in a number of ways, representative of which is the method developed at the New York Department of Health by Gibson,[22] Banzhaff, and others.[23] The original method consisted in

[22] Gibson. *Jour. Biol. Chem.*, Vol. 1, 1906.
[23] Gibson and Collins. *Jour. Biol. Chem.*, Vol. 3, 1907.

heating horse serum to 56° C. for 12 hours, by which some of the pseudoglobulin was converted into euglobulin, the antitoxin remaining in the pseudoglobulin fraction. After this an equal volume of saturated ammonium sulphate solution is added and the globulin precipitated. After several hours the precipitate is filtered off and again taken up in water corresponding in amount to the original volume of serum. After filtration this solution is precipitated with ammonium sulphate and this precipitate is treated with saturated solution of NaCl in quantity twice that of the original serum. After standing for 12 hours the supernatant fluid containing the antitoxin is decanted, and this is precipitated with 0.25 per cent. acetic acid. The resulting precipitate is dried by pressing it between filter papers and is placed in a parchment dialyzing bag, after neutralization with sodium carbonate. At the end of seven or more days of dialyzation against running water, the globulin solution remaining in the dialyzer is filtered and made isotonic.

More recently the method as modified by Banzhaff is as follows: The serum, as obtained from the horse, is diluted by one-half the volume of water, and to this a saturated solution of ammonium sulphate is added up to 30 per cent. saturation. This is heated to 61° C. for two hours. It is then filtered and the residue on the filter paper, which contains the antitoxin, is thoroughly dried by pressing between filter papers and is directly dialyzed.

Observations by Park and Throne [24] have shown that this concentrated antitoxin which, according to Gibson, represents a yield of about 70 per cent. original antitoxic power of the serum, is equally efficient for therapeutic purposes as an unconcentrated preparation and has the advantage of introducing less foreign protein into the human body. It retains its potency, according to Park and Throne, as long as does the whole serum.

Active Immunization in Diphtheria with Mixture of Toxin and Antitoxin

Recently Behring [25] has advocated the immunization of human beings with mixtures of diphtheria toxin and antitoxin. This method represents essentially *active* immunization with toxin rendered harmless by neutralization with antitoxin. The use of such mixtures had previously been studied with considerable care, in the case of the toxin of symptomatic anthrax, by Schattenfroh and Grassberger,[26] and the procedure had been used in the New York Department of Health for some years in the initial treatment of

[24] Park and Throne. *Amer. Jour. Med. Sci.*, Vol. 132, 1906.

[25] Behring. *Deutsche med. Woch.*, Vol. 39, No. 19, 1913.

[26] Schattenfroh and Grassberger. Deuticke, Wien, 1904; see also Schattenfroh, *Wien. klin. Woch.*, No. 39, September, 1913.

antitoxin horses. Theoretically considered on the basis of Ehrlich's opinions, one would be inclined to wonder at the fact that relatively neutral mixtures of toxin and antitoxin should possess any antitoxin-inciting properties. Behring explains the immunizing value of such mixtures by the reversible nature of toxin-antitoxin union in the animal body. He calls attention to the fact that our analyses of diphtheria toxin-antitoxin mixtures have been made entirely with guinea pigs as indicators. In studying such mixtures in other animals Behring has come to the conclusion that complete detoxication of the poison *in vitro* does not occur. He found, for instance, that a toxin-antitoxin mixture that was entirely innocuous for guinea pigs produced an active febrile reaction in an ass. In monkeys (*Macacus rhesus*) he finally found an animal in which he obtained evidence satisfactory to him that toxin may be powerfully active in the animal body, even if it has been previously mixed with antitoxin. If, for instance, he gave a monkey a mixture in which as much as 20 to 40 antitoxin units were mixed with one toxin unit, and repeated the injection two or three times, the animal died of subacute diphtheria toxin poisoning. The mixture ceased to be poisonous for monkeys only when the relation of antitoxin to toxin became one of 80 to 100 antitoxin units to one toxin unit. This final detoxication when sufficient amounts of antitoxin were used, it seems to us, may be taken as sufficient evidence that Behring's monkeys did not die of anaphylaxis.

We gather from Behring's writings that he attributes these differences in susceptibility to toxin-antitoxin mixtures in various animals to differences in the reversibility of the toxin-antitoxin complex in the bodies of the individual species.

Human beings are less susceptible to such mixtures than are monkeys, but nevertheless more so than guinea pigs. It also appears that diphtheria bacillus carriers or such persons who, because of a previous infection, have antitoxin in their blood are much more susceptible to these mixtures than are others. Newborn children are less susceptible than are children from 4 to 15 years. Mixtures which are entirely neutral for the newborn may incite febrile reaction in older children. In all cases the injection of such mixtures is followed by a more or less active production of antitoxin.

The mixtures which von Behring advocated were so prepared that the toxin action upon guinea pigs is practically nil; in other words, the mixture was completely neutralized.

The method represents in purpose, and apparently in achievement, a safe process of actively immunizing against diphtheria. Heretofore the method of protecting human beings prophylactically against diphtheria has consisted in the injection of antitoxic serum. This, unquestionably a wise procedure, has nevertheless the disadvantage of bringing about an immunity of short duration only.

Within 20 to 30 days the antitoxin injected may have completely or almost completely disappeared from the blood stream. Prophylactic immunization with the toxin-antitoxin mixtures, however, representing as it does an active immunization, is likely to be more prolonged in its effects. According to Behring a human being possessing 0.01 antitoxin unit in 1 c. c. of blood may be regarded as still moderately protected against diphtheria. According to his estimation a decline to this amount, in a person actively immunized by the mixtures (an estimation based upon curve measurements of treated cases), would take about two years. He has observed that horses that had been actively immunized by him, and subsequently used in agricultural work, retained measurable antitoxin values in their blood after five years without treatment.

Schreiber [27] and others state, also, that this method of active immunization with mixtures of toxin and antitoxin has the advantage of avoiding the anaphylactic dangers incident to the injection of antitoxin alone. Their opinion is probably erroneous, since it is most likely that whatever anaphylactic dangers there are result from the injection of horse serum rather than from the antitoxin contained in the injected substance. Moreover, the recent studies of Park have shown satisfactorily that the danger of anaphylaxis in the injection of antidiphtheritic sera is practically nil. Among 330,000 cases on record there were but five deaths.

The chief value of this new method of immunization is that it represents a safe technique for the prophylactic treatment of individuals exposed to the disease and possibly for the general prophylactic immunization of school children, nurses, physicians, etc. In the case of children during the ages at which they are most susceptible to the disease, the prolonged immunity resulting from the treatment should strongly recommend it as a method of promise for the gradual eradication of epidemics. Behring also suggests it as a hopeful method of treatment in the case of bacillus carriers.

Schreiber and others have reported upon the effects of treatment when carried out with Behring's mixtures. In the earlier experiments of Hahn, mixtures were used in which there was a slight excess of toxin. The later experiments were made with mixtures which were completely neutralized for guinea pigs. In Schreiber's cases from two to six injections were made at intervals of three to five days, most of them subcutaneously, and some of them intramuscularly. In no case were there serious reactions, although occasionally there were slight swelling of regional lymph nodes and a little fever. The effects of immunization were noticeable about 23 to 25 days later. When two injections only had been made, at least 0.075 of an antitoxin unit to the cubic centimeter was present. The highest value obtained after two injections was one unit to one cubic centimeter.

[27] Schreiber. *Deutsche med. Woch.*, Vol. 39, No. 20, 1913.

In nine patients who had been treated by four to seven injections with gradually increasing doses, as much as 10 to 75 antitoxin units to the cubic centimeter resulted. It appears, therefore, that in medical practice this method is safe, and that with as little as two injections antitoxin values may be obtained which entirely suffice for the protection of human beings against the ordinary dangers of diphtheria infection, an immunity which, as far as we can judge at present, may last about two years.

Another advantage which Behring claims for his method is the production of homologous antitoxin in human beings for the passive immunization of other human beings. Mathes has tried this in children with the idea of thereby avoiding the dangers of anaphylaxis. Incidentally it was claimed in this case that the passive immunization, when carried out with homologous serum, lasted longer than did that conferred by horse serum. However, one case is hardly enough to establish such a fact.

The Mixtures Actually Used.—The most extensive use of the Behring toxin-antitoxin immunization in diphtheria has been made by Park and his associates at the New York Department of Health. In carrying out this work, it has been found advisable from time to time to change the actual proportions of the mixtures used, as experience indicated the desirability of this. For a time the directions given for the mixtures used in this treatment were given as follows: the toxin and antitoxin used should be at least three months old, from the day of planting and the day of bleeding, respectively. The L_+ dose is determined with reference to the United States standard antitoxin. The unitage of the antitoxin to be used should be determined against this antitoxin, so that one unit of the antitoxin to be used, when mixed with the L_+ dose of this toxin, should permit the survival for 96 hours of 25 per cent. of the guinea pigs injected. When a tentative mixture of the product has been made, no further toxin should be added. Any changes made should consist, not in the addition of toxin, but in the reduction of toxicity by the addition of antitoxin, if necessary. Approximately, one unit of antitoxin is, therefore, added to start with, for each L_+ dose in the bulk of toxin, and with each addition of antitoxin to the mixture, it shall be immediately thoroughly shaken for 20 minutes. By repeated test and fractional addition of antitoxin, 0.1 of a unit at a time, the mixture is brought to a point where 5 c. c. injected into a pig, permits survival for 96 hours, and at this point the mixture is ready for filtering. After filtering, the mixture is preserved until at least one-half of the 5 c. c. guinea pigs on each bulk bottle, survive 10 days, but show definite paralysis. The human dose of this was 1 c. c.

Park, Schroder and Zingher [28] have recently changed this mix-

[28] Park, Schroder and Zingher. *Jour. Amer. Pub. Health Assoc.*, No. 13, 1923, p. 23.

ture in the direction of less toxicity and, therefore, less danger of severe reaction. They experimented with various mixtures, in all of which there were different amounts of toxin and antitoxin, but all of them striking such a balance between the two, that 1 c. c. caused paralysis in 300 gram guinea pigs, and 5 c. c. caused death in guinea pigs within 10 days. These mixtures were made on the basis of Park's statement that the best mixture is one that is underneutralized, and yet perfectly safe. As the mixture stands the toxin deteriorates faster than the antitoxin, so that it gradually deteriorates in usefulness. Apparently annoying reactions are obtained because of protein reactions due to diphtheria bacillus cell substances found in the toxin. In using mixtures such as those described, in which the balance between toxin and antitoxin was the same, but the actual total amount of toxin was reduced, they found that the different preparations gave the same immunizing results, but those having the least amount of toxin and, therefore, the least amount of accompanying bacillus substance, showed the least amount of reaction. The following table published by Park and his associates gives results obtained with mixtures made up in this way.

TABLE II[29]

The antitoxin development produced by three injections of mixtures having different amounts of toxin and antitoxin but all causing severe paralysis in guinea pigs receiving doses of 1 c. c. and death within ten days in those receiving 5 c. c.

Amount of Original Toxin in 1 c.c. of Mixture	No. of School Children Receiving 3 Injections	Per cent. of Nonimmunes Shown To Be Immune on Schick Retest 4 Months Later
*1/10 L + (4 lethal doses).....	490	90%
½ L + (20 lethal doses).....	304	95%
3 L + (120 lethal doses).....	318	92%
5 L + (200 lethal doses).....	487	85%

* The mixture is made by adding three-fourths of a unit of antitoxin to one L + dose of toxin. The toxin and antitoxin should be diluted in cold water and the two solutions mixed immediately. If the toxin is diluted in water at room temperature it deteriorates rapidly.

TABLE III

Comparison as to the amount of local and constitutional reaction caused by the new and old preparation.

	* New Preparation 1/10 L +	Old Preparation 3 to 5 L +
No Local Reaction......................	25%	0%
Slight Local Reaction...................	64%	41%
Moderate Local Reaction................	11%	37%
Marked Local Reaction..................	0%	22%
Of those showing marked reactions there was a rise of 1° to 3° F., and other constitutional symptoms in................	0%	6%

* If the 1/10 L + preparation is underneutralized there will be a local reaction from the excess of toxin.

[29] Taken directly from Park, Schroder and Zingher, *loc. cit.*

The Intracutaneous Method of Determining Toxin and Antitoxin Values [30]

Marks [31] was the first to utilize the prevention of local edema or injury for the determination of antitoxin values. He mixed diphtheria antitoxin and toxin and injected them subcutaneously into guinea pigs, claiming that this method was considerably more delicate than the Ehrlich method, since the amount of toxin capable of causing localized edema amounted to as little as one-twentieth of a minimal lethal dose. This method has many points in its favor, and has been recently utilized and improved upon by Römer.

Römer [32] has developed a method of diphtheria antitoxin standardization which depends upon intracutaneous injections into guinea pigs. The principle of this test consists in the observation that, when very slight amounts of diphtheria toxin are injected intracutaneously into the abdominal skin of guinea pigs, small areas of local necrosis result within about 48 hours. When such injections are made with mixtures of toxin and antitoxin the presence of free toxin is indicated by the appearance of such necrosis.

Before proceeding to the standardization by this method it is necessary to determine the "limes-necrosis" (just as Ehrlich determines his L_+ dose), that is, the amount of toxin which, together with a given amount of toxin (1/50, 1/200, or 1/2,000), will still produce a minimal amount of necrosis after intracutaneous injection into guinea pigs. It is necessary, therefore, arbitrarily to choose a certain definite fraction of an antitoxin unit and mix this with varying amounts of toxin and inject the mixtures into guinea pigs intracutaneously. Those mixtures in which the toxin is fully neutralized will give rise to absolutely no lesion further than, possibly, a slight local edema. Those in which there is a large excess of toxin will cause extensive necrosis. Between the two, in the series, there will be a mixture in which slight local necrosis results from the injection. In this mixture the amount of toxin, just sufficient to cause noticeable necrosis in spite of admixture with the antitoxin, contains the L-n (limes necrosis) dose.

When this has been determined, then unknown antitoxin can be similarly measured against this L-n dose of the standard toxin. The method has the advantage of permitting one to work with very small quantities, since only a small fraction of a cubic centimeter need be used for intracutaneous injections; also it permits great economy of

[30] See also Kellogg's method described on p. 522, under the discussion of methods for the determination of antitoxin in blood serum of human beings.

[31] Marks. *Centralbl. f. Bakt.*, Orig. Vol. 36.

[32] Römer. *Zeitschr. f. Imm.*, Vol. 3, 1909, p. 208. Römer and Sames. *Ibid.*, p. 344. Römer and Somogyi. *Ibid.*, p. 433.

animal material, since four or five tests can be simultaneously carried out upon the abdominal wall of the same guinea pig.

The technique is not easy. We have found in studying this method in connection with some work carried on in our laboratory by Dr. M. C. Terry, that a considerable amount of practice and experience is necessary, both in carrying out the procedure accurately and in judging the lesions. However, when carefully and consistently done by an experienced worker, this method gives results which correspond with fair accuracy to measurements made of the same antitoxin by the Ehrlich method. This has been the experience of Lewin,[33] and also of Terry in the few experiments carried out by him.

The Römer method has been recently used by clinicians for the determination of the presence of free toxin or antitoxin in the circulating blood of patients suffering or convalescent from diphtheria. Römer himself suggested this, since his method is adapted to the determination of extremely slight amounts of either substance. A recent study by Harriehausen and Wirth [34] illustrates the results obtained in such tests. Normal human serum injected intracutaneously into guinea pigs never caused necrosis. Neither did the similar injection of the sera of children suffering from varicella and other diseases. Of twelve children suffering from diphtheria, however, serum taken before the administration of antitoxin caused necrosis upon intracutaneous injection into guinea pigs, in every case. In spite of the administration of antitoxin, toxin was demonstrable in the blood in five cases as long as the 35th day. Of ten cases of post-diphtheritic paralysis, toxin was demonstrated in the blood of five.[35]

Schick Reaction.—In 1912 Michaelis and Schick [36] carried out intracutaneous reactions with diphtheria toxin directly upon human beings to determine whether or not diphtheria immunity was present. Their early experiments were carried out by injecting 0.1 c. c. of a 1-1,000 dilution of toxin, and their results indicated that a positive intracutaneous reaction with this amount indicated an absence of antitoxin from the blood, or at any rate, an insufficient protection. The reaction was called positive when within 24 to 36 hours there appeared a slight infiltration of the skin surrounded by a red areola 1 to 2 cm. in diameter. The reaction may continue to increase, or in weak positives may not come out completely for 48 hours, thereafter fading gradually. This reaction has attained great importance in public health work since its first introduction. At the present time it is being carried out on many thousands of children and

[33] Lewin. *Centralbl. f. Bakt., Orig.,* Vol. 67, 1913.
[34] Harriehausen and Wirth. *Zeitschr. f. Kinderheilkunde,* Vol. 7, 1913.
[35] See Kellogg's modification, p. 522.
[36] Michiels and Schick. *Zeitschr. f. Kinderheilkunde,* Vol. 5, 1912.

adults in the United States and Europe. In America it has been studied chiefly by Park and Zingher.[36a]

The material used for the reaction at the present time consists of a standardized diphtheria toxin, diluted in such a way that 0.1 c. c. contained 1/50 M L D for a guinea pig. A very large number of tests has indicated that this amount will produce no reaction on intracutaneous injection into a human being if the blood serum of the subject contains more than 1/30 unit of antitoxin per cubic centimeter. Park and Zingher have found that negative reactions were present in about 93 per cent. of the newborn, and became less frequent up to the fifth year at which time 37 per cent. were negative. In our own experience with the Army in an age group ranging from 20 to 30 years, an average of about 10 per cent. positives was obtained.

In performing the reaction a certain number of precautions must be carefully observed. A pseudo reaction may appear a little earlier than the true reaction, and disappear within 24 to 48 hours. This is occasionally seen in individuals who may have sufficient antitoxin but who are sensitive to either the bacterial protein or the cultural material present in the injected toxin. It may be obtained in the same individual by the injection of autolyzed diphtheria bacilli, and sometimes by the injection of uninoculated broth. To avoid error, therefore, it is necessary, when doing Schick reactions, to carry out a control injection consisting of 1/50 M. L. D. of the toxin heated to 80° for 5 minutes. This destroys the toxin, but leaves uninjured the substance which produces the pseudo reaction.

Tetanus Antitoxin and Its Standardization

The methods employed in the production and standardization of tetanus toxin are in every way analogous to those used in the case of diphtheria antitoxin. A strong toxin is obtained by growing the organisms under anaërobic conditions on suitable media. According to Vaillard and Vincent[37] it is essential that the media upon which the tetanus bacilli are grown should be freshly made and sterilized. Apparently this precaution, which has been similarly recommended by Wladimiroff, Novy, and others, is made necessary by the gradual absorption of oxygen which takes place if the media are allowed to stand for a long time without heating. It is further necessary in preparing tetanus toxin that the culture medium should not be acid, and a weakly alkaline initial titre is advised. For the same reason, also, most workers have advised against the use of glucose or other carbohydrates in the media, since the acid formed by the fermentation of these substances inhibits growth and toxin

[36a] Park and Zingher. *Jour. A. M. A.*, 65, 1915, p. 2216.
[37] Vaillard and Vincent. *Ann. de l'Inst. Past.*, 1891.

production. Recently Hall [38] has advised the use of a simple meat extract broth to which have been added 1 per cent. of dextrose and 0.5 per cent. of finely powdered magnesium carbonate. The last-named substance, by neutralizing any acid that is formed from the glucose, prevents the harmful acidity. Anaërobic conditions are obtained by growing the organisms under a layer of oil in tightly stoppered flasks.

Although mice were formerly used in the standardization of tetanus toxin and antitoxin, the more recent usage has been to substitute guinea pigs as in diphtheria standardization. According to the recent directions of Rosenau and Anderson [39] the purposes of the standardization are carried out as follows:

The unit of antitoxin is arbitrarily designated as 10 times the smallest amount of serum necessary to preserve the life of a guinea pig weighing 350 grams for 96 hours, when given together with an official test dose of toxin. The test dose of toxin contains 100 minimal lethal doses. And the minimal lethal dose is measured against a 350-gram guinea pig.

In carrying out the standardization the L+ dose of the toxin is used, but, unlike diphtheria standardization, in this case the L+ dose means an amount of toxin which will kill a guinea pig of 350 grams in four days, although united with 0.1 unit of antitoxin (it must be noted that the L+ dose in this case is measured against one-tenth unit of antitoxin rather than against 1 unit, as in the case of diphtheria).

In determining the value of an unknown antitoxin, mixtures are made, each containing the L+ dose of the toxin and varying quanties of antitoxin. As in diphtheria measurements, the various injection volumes are brought to 4 c. c. with salt solution, and are then injected subcutaneously into guinea pigs of about 350 grams. The table given below is taken from the Bulletin of Rosenau and Anderson.

No. of guinea pig	Weight of guinea pig (grams)	Subcutaneous injection of a mixture of		Time of death
		Toxin (Test dose) (gram)	Antitoxin (c. c.)	
1	360	0.0006	0.001	2 days 4 hours
2	350	0.0006	*0.0015*	4 days 1 hour
3	350	0.0006	0.002	Symptoms
4	360	0.0006	0.0025	Slight symptoms
5	350	0.0006	0.003	No symptoms

[38] Hall. "Univ. of Cal., Publ. in Path.," Vol. 2, No. 11, 1913.
[39] Rosenau and Anderson. *U. S. P. H. Service Hyg. Lab. Bull.* 43, 1908.

In this experiment 0.0015 equals 0.10 antitoxin unit.

Therapeutic Use of Tetanus Antitoxin.—Prophylactically, tetanus antitoxin has always been used with a great deal of success, and its efficiency was established beyond question of doubt during the recent war. Its curative value, however, has always been considerably limited, probably owing to the early union of tetanus toxin with cells of the central nervous system. But recent improvements in the methods of administration have considerably increased its efficiency.

As a general rule, for *prophylactic use,* subject, of course, to individual modifications in various types of cases, the following mode of procedure is advisable. About 1,000 to 1,500 units are administered subcutaneously, and this dose repeated if secondary injury or operation on the wound. It may be said with reasonable certainty that tetanus antitoxin after injection disappears in the human being almost completely in the course of 2 or 3 weeks, and in cases, especially of compound fracture in which operative interference is called for within this time, one cannot rely upon a holding over of the protective effect of the first injection. In children, of course, proportionately smaller doses should be given. A similar repetition of the dose may be advisable when inflammatory conditions in the wound, with discharge, etc., continue over a prolonged period. All wounds made by implements soiled with dirt, especially garden earth in which horse or cow manure or other faecal material may be suspected, or in which any severe contamination of the wound is likely to have occurred, and especially those in which there has been extensive tissue destruction, laceration, are to be suspected of tetanus. Especially is this the case in gun-shot and shell wounds, in which particles of soiled clothing are carried into the wound with the projectile. In the last war it was a custom to inject every wounded man with tetanus antitoxin as soon as he came under medical observation, and there is little question that this procedure saved many lives.

For curative purposes we follow the advice given by Matthias Nichol, Jr.,[40] who has worked on this problem, both experimentally and practically, at the New York Department of Health. In the curative use of the antitoxin, the speed of treatment is one of the most important factors for success. He states that every hour lost after the development of symptoms makes the prospect of success less bright. Every case coming under observation with symptoms suspicious of tetanus, according to Nichol, should receive an intraspinous dose of tetanus antitoxin of not less than 10,000 units, and this dose should be repeated in 12 hours, and again in 24 hours if the symptoms continue. At the same time the intraspinous dose is given, Nichol advises an intravenous dose of 5,000 to 10,000 units.

We have seen one or two cases, also, in which there seems to have

[40] Nichol, M. *Jour. A. M. A.,* Vol. 76, 1921, p. 112.

been some benefit by adding to this an injection of small amounts of antitoxin in the neighborhood of the wound, in the region of the afferent nerve trunks. Whether or not this is of value, however, is questionable. Nichol believes that the intraspinous use is the most important application, and advises the use of a general anesthesia when opisthotonos is present, or when any condition exists which makes a very careful and deliberate operation difficult. The antitoxin should never be forced into the canal, and a certain amount of fluid can be withdrawn through the same needle before the antitoxin is injected, if the spinal fluid is under pressure. Nichol also advises diluting the antitoxin with sterile salt solution to a total of about 15 c. c. in the case of children, and 30 c. c. in the case of adults. The intraspinous administration of the serum is subject to no greater danger than the intravenous administration, according to this worker.

ANTITOXINS AGAINST SNAKE POISONS

(Antivenin)

Antitoxins against snake poisons have been produced by a number of different workers, but the subject has been most extensively studied by Calmette. As early as 1887 Sewall [41] succeeded in increasing the resistance of pigeons to snake poison. Later Calmette and Physalix and Bertrand independently succeeded in producing immunity in rabbits and guinea pigs with the poison of the cobra. The serum of animals treated with snake poisons gradually acquires antitoxin properties, but the process of immunization is not a simple one, and considerable time is needed for the immunizations.

Snake poisons, as we have seen, have attracted considerable attention because of their peculiarities in being antigenic and yet differing in heat resistance and a number of other properties from the bacterial toxins. It was with snake poisons that Calmette definitely showed that the union of toxin and antitoxin is a true neutralization and is not accompanied by the destruction of the toxin. These experiments, as we have seen, were elaborated later by Morgenroth, who succeeded in producing the snake poison HCl combination. It is these poisons also that have been the subject of extensive study by Flexner and Noguchi, by Kyes, and later by von Dungern and Coca. This work has been sufficiently discussed in other places and need not occupy us here. The important poisonous snakes may be divided into the colubridæ, to which class the cobra belongs, and the viperidæ, which includes the ordinary European vipers, the rattlesnake, and most of the poisonous snakes of North and South America.[41a] Ac-

[41] Sewall. Cited from Calmette.

[41a] There are at least two other varieties of poisonous snakes in India against the poisons of which no antitoxic sera have as yet been successfully produced.

cording to Calmette the poison of the cobra is much more heat-stable than that of the rattlesnake. Pharmacologically the poisons of these two main classes of snakes show considerable difference. In the case of the cobra there is very little local disturbance and the systemic symptoms dominate the clinical picture. Calmette describes the cobra bite as being followed only by a feeling of stiffness at the site of the bite, followed very soon by great general weakness, difficulty in respiration, slow heart action, and finally death with unconsciousness. In the case of the vipers the local symptoms are very much more marked, there being great pain and swelling and apparent clotting of the blood about the point of the bite, with a rather slower onset of systemic symptoms. In a description by Sparr [42] of a case of bite by Russell's viper there was almost immediate swelling of the limb with a faint bluish tint around the pin-point puncture, and within 15 minutes great weakness, restlessness, and retching. In spite of very active local treatment, within a short time after the bite, the patient died within less than 24 hours of asphyxia and heart failure.

According to Calmette 0.0002 gm. of cobra poison will kill a guinea pig; Noguchi states that 0.0005 gm. of rattlesnake venom will kill a guinea pig of 250 gr. within 24-30 hours when injected intraperitoneally. The snake poisons apparently contain substances which are especially active upon nerve cells (neurotoxins), and hemolysins which act particularly upon the red blood cells. Flexner and Noguchi [43] also speak of another poison which acts particularly upon the endothelium of the blood vessels producing hemorrhages.

According to Calmette the antisera which are produced by immunization with cobra poison are most strongly potent against neurotoxic poisons of the colubridæ and, to a certain extent, against some of the poisons of the vipers. However, the action of the cobra antitoxin against viper poison seems at best to be weak. On the other hand, antitoxins produced with rattlesnake poison are not potent against the cobra venom since, as Calmette states, the rattlesnake poison contains hardly any neurotoxin. Antitoxins may be produced by the gradual immunization of horses, and have been produced in this way by Calmette in the Pasteur Institute of Lille for some years. Calmette standardizes his antitoxin by determining the amount of serum which completely neutralizes *in vitro* 0.0001 gm. of the poison as tested upon white light. He also determines the protective power by injecting a rabbit with 2 c. c. of the serum and two hours later gives 1 gm. of the poison.

Noguchi has studied rattlesnake poison particularly and succeeded in preparing a strong antitoxin by the gradual immunization of a goat. Great difficulty has always been experienced in attempts

[42] Sparr. *Biochem. Bull.*, Dec., 1911, No. 2.
[43] Flexner and Noguchi. *Univ. of Pa. Bull.*, Vol. 15, 1902.

at immunization with rattlesnake poison because of the very violent local injury produced by injections of the venom. The potency of the serum produced by him was such that 2½ c. c. of goat serum protected guinea pigs against 12 times the fatal dose of rattlesnake poison if given at the same time. If the antivenin was given one hour later, 5 times the amount of serum had to be given.

Serum Treatment in Botulismus.—Antitoxic serum has been produced by the injection of botulinus toxin by a number of observers. Kempner [44] started research in this direction in 1897. He was followed by Jorssman and Lundstrum,[45] and Leuchs.[46] Dickson and Howitt [47] have recently produced potent antitoxins by the immunization of goats. These observers made the important observation that various strains of B. botulinus produces at least two different toxins which are not serologically homologous. Representatives of these two types must be used in the production of the antitoxin.

There is not yet sufficient statistical evidence to permit us to draw conclusions concerning its therapeutic value. Dickson advises its use, however, by the ordinary methods of intravenous injection.

Antitoxin Treatment in the Case of Anærobic Wound Infection. B. Welchii Toxin.—Since, in 1917, Bull and Pritchett [48] were able definitely to determine the presence of a toxin in cultures of the Welch bacillus, the possibility of antitoxin treatment of infections with this organism was established. Before this time, a suspicion that the Welch bacillus produced a toxin had often been suggested but never definitely proven. At about the same time that Bull and Pritchett made their observations, similar observations were made by Weinberg and Seguin.[49] Klose [50] had reported similar observations in 1916, but the toxin produced by him was not very powerful, and the antitoxin resulting from its injection into animals gave limited protection only. Apparently, the production of the toxin in the cultures of Bull and Pritchett depended upon the presence of fresh muscle tissue and glucose in the broth in which the organism was grown. They found no variations in the different strains of B. Welchii, irrespective of the source, to produce the toxin, but toxin production seemed to be directly proportionate to virulence. Most potent toxin production seems to be obtained by inoculating the infected muscle of a pigeon dying of B. Welchii infection directly into the medium to be used for toxin production. Caulfield [51] who has

[44] Kempner. *Zeit. f. Hyg.*, Vol. 26, 1897, p. 481.
[45] Jorssman and Lundstrum. *Ann. de l'Inst. Past.*, Vol. 16, 1902, p. 294.
[46] Leuchs, Kolle and Wassermann. *Handbook*, 2nd Edit., Vol. 4, p. 939.
[47] Dickson and Howitt. *Jour. A. M. A.*, Vol. 74, 1919, p. 718.
[48] Bull and Pritchett. *Jour. Exp. Med.*, Vol. 26, 1917, p. 67.
[49] Weinberg and Seguin. "La Gangrene gazeuse," Masson, Paris, 1918.
[50] Klose. *Münch. med. Woch.*, Vol. 63, 1916, p. 723.
[51] Caulfield. *Jour. Inf. Dis.*, Vol. 27, 1920.

studied this, claims that good toxin production does not result unless the virulence of the strain is such that 0.02 c. c. of the supernatant fluid of a brother culture will kill a 300 gram pigeon. Work by Bengston of the Hygienic Laboratory in Washington, however, and similar work by DeKruif seems to indicate that the substitution of chopped veal in broth, even after autoclaving, furnishes a favorable medium for toxin production. The toxin differs from the classical toxins in that it varies in potency with virulence of the strain, and has no definite incubation period.

Antitoxin is produced by the injection of horses, and an attempt to standardize the antitoxin has been made by Bengston, who succeeded in producing an antitoxin of which 1 c. c. of serum contains one unit, one unit neutralizing 1/1,000 of an M. L. D. of the B. Welchii toxin.

In laboratory animals injected with pure cultures, the antitoxin gives complete protection. Similar protection, however, seems to be provided, according to Weinberg and Seguin, by antibacterial sera prepared by the injection of whole broth cultures of the bacillus, although the antitoxin contents of these sera has not been determined.

The usefulness of the serum in human beings has not been sufficiently tested for final judgment, but there is no theoretical reason why it should not be very valuable in infections by pure cultures of the Welch bacillus.

Unfortunately, most wounds that contain Welch bacillus are apt to contain other anaërobic organisms, particularly the Vibrion septique and the B. cedematiens. Efforts, therefore, to produce mixed antitoxins are being made.

Antitoxin Against Vibrion Septique.—The Vibrion septique, according to Weinberg and Seguin,[52] occurred in about 12 per cent. of the wounds examined by them. It is rarely alone, but usually associated with other anaërobes, particularly the Welch bacillus.

All strains of the Vibrion septique produce a powerful toxin. This substance, too, is peculiar in that it kills without an incubation period comparable to that noticed in the case of most other toxins. It is peculiar, also, in that death occurs with regularity only when the substance is injected intravenously, and often when injected subcutaneously or intramuscularly produces a local necrosis only.

A powerful toxin may be obtained by growing the organism anaërobically in an 0.2 per cent. dextrose broth to which 10 per cent. of horse serum has been added. Robertson [53] has recommended the addition of liver tissue from a guinea pig dead of Vibrion septique, for inoculation into broth. Great care has to be exerted in controlling filtration, since filters which are too tight hold back a considerable percentage of the toxin.

[52] Weinberg and Seguin. *Loc. cit.*
[53] Robertson. *Jour. Path. and Bact.*, 1920.

Antitoxin has been prepared by Weinberg and Seguin, and by Robertson, by injecting the toxin into horses and sheep. The development of the antitoxin has been undertaken chiefly in France where the standard requires that 0.001 c. c. of the antitoxin should neutralize two fatal doses of the toxin after 30 minutes' incubation of the mixture at room temperature and injection into guinea pigs.

Bacillus Œdematiens.—Weinberg and Seguin isolated this organism in 34 per cent. of the war wounds examined. The percentage of most other workers has been lower. The organism and the toxin described are probably identical with what Sacquepee [54] has spoken of as the B. Bellonensis. This organism forms a soluble toxin which is of unusual strength. It is best produced, according to Weinberg and Seguin, by growing the organism in broth, containing chopped veal, for about a week. It never kills acutely on intravenous injection, in this respect differing from that previously described, but with a potent strain, 0.01 c. c. of the broth filtrate, intravenously injected, will kill a 300 gram guinea pig in 48 hours.

Rabbits, sheep and horses, carefully injected with broth filtrates from these strains, will eventually yield an antitoxin of considerable strength. Weinberg and Seguin, using horses, prepared an antitoxin of which 0.0001 c. c. neutralized two lethal doses for a guinea pig.

Passive Immunization in Bacillary Dysentery.—In view of the fact that a soluble toxin has been claimed by a number of workers for the Shiga dysentery, and that animals immunized with organisms of the Flexner group yield a serum to some extent neutralizing the toxic effects of these organisms, sera have been produced by the immunization of horses with organisms of both the Shiga and the Flexner type. Flexner advises the separate immunization of horses with these organisms, and the use of the sera either mixed or separate as indicated by the bacteriological examination of the cases.

When serum has been employed, it has usually been used subcutaneously, but it may be used intravenously in quantities ranging from 20 to 100 c. c., according to the age of the patient and the severity of the case. According to Flexner,[55] a single injection has often led to marked alleviation of symptoms, the most striking results having been obtained in acute cases; but favorable results have also been obtained in the second and third weeks. The same writer states that the mortality in certain outbreaks in which comparisons between serum treated and untreated cases have been made, was reduced from 10 to 15 per cent. to 1 to 2 per cent. under serum treatment. And in epidemics in which the mortality reached 50 per cent., serum treatment has reduced the mortality to about 10 per cent.

In institutional outbreaks of the disease he advises prophylactic doses of 5 c. c., subcutaneously injected.

[54] Sacquepee. *Ann. de l'Inst. Past.*, Vol. 30, 1916, p. 76.
[55] Flexner. *Jour. A. M. A.*, Vol. 76, 1921, p. 108.

PASSIVE IMMUNIZATION IN DISEASES CAUSED BY BACTERIA WHICH DO NOT FORM SOLUBLE TOXINS

General Principles.—As we have stated, the greatest therapeutic successes with passive immunization have been achieved in bacterial diseases in which the malady is essentially a toxemia due to a toxin. In such cases the serum of actively immunized animals contains specific antitoxins by virtue of which the toxins circulating in the blood of the patient are directly neutralized, quantity for quantity, with consequent therapeutic benefit. In the case of bacteria in which no toxins are formed, the immunization of an animal is not followed by the formation of any poison-neutralizing principle. Here the injection of bacteria, dead or alive, or the invasion of the bacteria in the course of spontaneous disease, is followed by the formation of specific antibacterial substances, lytic, opsonic, agglutinating, or precipitating bodies, the nature of which we have discussed in other chapters. The toxemia which occurs in such cases is due as we have seen to derivatives of the bacterial protein which by some observers are regarded as preformed endocellular poisons liberated by the lytic action of the serum, and by others as split products of the bacterial protein, non-existent until the bacterial cell has been acted upon by the serum components and destroyed. However this may be, the recovery from diseases of this nature is accomplished by bacterial destruction; this may be rarely, by the bactericidal action of the serum, but more often by opsonic powers which induce phagocytosis. The poisons which are liberated from the bacterial bodies, if free, can do their injury, and no neutralizing substance is formed in the body fluids to prevent their action as far as we know. Immunity in such cases, then, is not an antitoxic immunity in any sense of the word; it is rather an antibacterial immunity in which the disease is prevented or cured only when complete destruction of the bacteria has taken place. If an animal or a human being is prophylactically immunized against diseases of this kind (typhoid, cholera, etc.), it is easy to see that an increased presence of antibacterial substances, bactericidal or opsonic, in the circulation would serve efficiently and rapidly in disposing of the small numbers of invading micro-organisms which ordinarily enter the body in spontaneous infections. And, indeed, experience has shown that prophylactic immunization can be successfully carried out in the case of cholera, typhoid fever, plague, and other diseases which are sufficiently prevalent endemically or epidemically to justify prophylaxis on an extensive scale.

However, when in diseases of this kind the body is already extensively infected and has begun, as is usually the case, to respond spontaneously with the formation of specific antibodies, it has been a matter of doubt whether or not passive immunization, that is, the

introduction of specific antibodies in the form of the serum of a highly immunized animal, is therapeutically of value. Indeed, it has been feared that the use of such sera may even be harmful in that the sudden introduction of large amounts of bactericidal substances might lead to a sudden liberation of large quantities of poisonous products and consequent rapid toxemia.

The conditions in such cases are exceedingly complex and many gaps exist in our knowledge concerning them. The bacteria when invading the body, immediately enter into conflict with the protective forces, as we have stated in the chapter on Infection. If a considerable degree of resistance exists, let us say as the result of preceding immunization or a recent attack of the disease, there is a rapid destruction of the bacteria, probably by active phagocytosis. It has been shown by Bordet in the case of cholera and more recently by Gay with typhoid, that injection of the organisms into immunized animals is followed by prompt and high leukocytosis, whereas similar injections into normal animals usually induce a temporary leukopenia. When the invaded animal is not particularly resistant the bacteria may accumulate and, as in the case of pneumococci and streptococci, develop phagocytosis-resisting properties (capsule formation, etc.); or, as in the case of typhoid bacilli, there may be an immediate liberation of toxic substances (endotoxins, etc.) by reaction between bacterial cell and blood plasma, which can induce leukopenia, and by this means the organisms may be protected from phagocytic destruction. Experience with curative sera in all of the conditions of this class has yielded promising results only when the cases have been treated with the sera at early stages of the disease, either when the invading germ was still localized or, at least, when the septicemic condition was not yet thoroughly established. It may be that the doses heretofore given have been insufficient, and indeed recent experiences with pneumonia seem to indicate that this may have been, in part, the cause of earlier failures. Yet in pneumonia the septicemia probably does not represent the firm establishment of a foothold by the pneumococcus in the circulation but rather a continuous discharge of new organisms into the blood from the localized lesion in the lung.

It is our own opinion, moreover, that septicemia as usually observed clinically represents in most cases exactly this condition, that is, a more or less continuous discharge of the bacteria into the blood from some active focus with a continuous destruction of the organisms after they have entered the blood stream. It is only when the resistance of the body is overwhelmed, in the later stages of the disease, that the bacteria can continue to grow and develop in the circulation, and this stage probably does not occur until death is imminent. In such septicemic diseases as streptococcus infection, typhoid fever, plague, anthrax, and many others the presence of the

bacteria in the blood at the time when the patient is still in a condition of powerful resistance probably means that the bacteria are being supplied to the blood from the local lesions. There is probably just such a continuous discharge of bacteria from the focus into the blood with active destruction after the bacteria have entered the circulation. This seems especially probable from the fact that in many of these diseases the protective antibodies, bactericidal and opsonic, can often be demonstrated in the blood serum in quantities higher than normal at the very time when blood culture yields positive results. In typhoid fever, of course, it is well known that bactericidal titres of over 1-50,000 are often present while the patient may still be very sick, and in the more chronic streptococcus conditions with malignant endocarditis we have often seen that opsonic properties on the part of the patient's serum against the very organism invading him are considerably higher than normal. We take this to mean that the injection of immune sera would simply aid in more rapidly freeing the blood stream of the bacteria, the cure of the disease, however, involving a destruction of the focus. This, of course, is not possible merely by the injection of the serum. When, as in some cases of streptococcus infection, the focus can be surgically reached, the septicemia will often disappear and cure result, as we have ourselves had the opportunity to observe. When the focus cannot be reached surgically, it may nevertheless be a wise procedure to inject considerable amounts of immune serum, for, by keeping the blood stream free of bacteria, the case may be influenced favorably. Pneumonia is an example of this. Former failures have recently been turned into partial success by the work of Neufeld and of Cole merely by the use of larger quantities of immune sera essentially similar to sera used at previous times, and Cole attributes the apparently favorable results to the fact that the blood stream can be cleared of bacteria although the focus cannot itself be affected.

Rapid and complete cure of such diseases, therefore, can hardly be expected. Favorable influence of the disease by energetic serum treatment may, however, be hoped for. As to the exact manner of action of such sera there is much room for discussion. It is likely that a number of factors are involved. Chief among them is probably the increased opsonic power of the patient brought about by the specific sensitization of the bacteria. In addition to this there is, in many cases an active intravascular agglutination of the bacteria, as shown by Bull for animals infected with pneumococci and typhoid bacilli. By this the bacteria are clumped in the capillaries of some of the viscera where active phagocytosis by fixed tissue cells can then take place. A certain amount of actual neutralization of so-called "endotoxic" substances by the antibacterial sera has also been claimed in the cases of sera prepared with Gram-negative organisms like the typhoid and Dysentery bacilli and the meningococcus.

In discussing this subject it must not be forgotten, however, that in most of the diseases which we have classified, on the basis of prevailing opinions, as caused by bacteria that do not form true toxins, the formation of such poisons has been claimed by a number of careful and eminent observers. In the case of the typhoid bacillus, especially, Chantemesse, Kraus and Stenitzer, and others have claimed the existence of a true toxin and a consequent antitoxin in immune sera. Similar claims have been made for the cholera spirillum by Kraus and Doerr, for the streptococcus by Marmorek, and for the plague bacillus by Markl and Rowland. Since these claims have been made on the basis of extensive experimentation by competent men the question must be left open, and the possibility of antitoxic properties on the part of the sera cannot be completely ignored. Since in most cases, however, the poison-neutralizing properties of the immune sera in this disease have not exceeded more than 1 to 2 multiples of the M L D of the bacterial poisons, it does not seem impossible that the apparent antitoxic properties may have represented merely an acquired tolerance to anaphylatoxic poisons of which we have spoken in another place.

Serum Treatment in Epidemic Cerebrospinal Meningitis

Serious attempts to produce curative sera against the epidemic form of cerebrospinal meningitis were not made until 1906 and 1907, when this disease appeared epidemically chiefly in Europe, where it appeared most severely in Eastern Germany, and in the Eastern United States.

In 1906 Kolle and Wassermann immunized three horses with meningococci, using for immunization purposes the dead organisms followed by living cultures and cultures taken up in distilled water, the so-called artificial aggressins of Wassermann and Citron. They obtained sera of considerable potency when measured against meningococcus cultures, and suggested standardizing the sera by complement fixation. They did not at this time treat human beings, but suggested the use of the serum subcutaneously and intravenously in meningitis cases. Very soon after the publication of the work of Kolle and Wassermann Jochmann [56] also produced an antimeningococcus serum by immunizing horses with proved meningococcus cultures, in his cases making a polyvalent serum by the use of many different strains of the organism. The sera which he obtained were highly agglutinating, somewhat bactericidal, and, according to him, not antitoxic. He first succeeded in immunizing guinea pigs against meningococci by injecting the serum 20 hours before infecting the animals. He also treated 40 cases of meningitis in man and ob-

[56] Jochmann. *Deutsche med. Woch.*, Vol. 32, 1906, p. 788.

tained encouraging results in cases treated before the development of hydrocephalus. Believing that possibly intraspinous injection of the serum might offer advantages, he first determined by experiments upon the dead body that the injection of methylene-blue intraspinously passed from the point of injection in the lumbar regions as far up as the olfactory nerves. After having determined this he treated 17 cases by tapping the spinal canal, taking out 30 to 50 c. c. of spinal fluid and then injecting about 20 c. c. of the serum. Of these 17 cases only 5 died, and Jochmann expresses himself optimistically in consequence.

Meanwhile Flexner [57] had been working upon the same subject, laying a rather more thorough basis for therapy in careful animal experimentation. He produced the typical disease in monkeys by intraspinous inoculation of the meningococci and then saved the animals from death by following the infection with the injection of serum intraspinously six hours later. In his earlier articles he expresses himself with much conservatism, but his studies were continued and extensive opportunity for testing the serum which he then produced, together with Jobling,[58] was offered by the continuance of the epidemic throughout the United States.

In 1908 Flexner and Jobling reported upon 47 cases treated with the antiserum of which 34 recovered. Of 12 additional cases reported in an addendum only 4 died. Flexner [59] records of 1,294 cases that have been treated with the serum prepared at the Rockefeller Institute. Of this number, unselected and treated in many different parts of the world, 69.1 per cent. recovered. It is of course very difficult to obtain exact comparative data on the efficiency of any method of treatment in a disease as irregular in its clinical manifestations as epidemic meningitis, especially since the mortality attending upon different epidemics is subject to great variations. For this reason we can draw conclusions only from a large statistical material. However, we know that the average mortality of epidemic meningitis before the introduction of specific therapy ranged certainly higher than 65 per cent., and in carefully studied epidemics usually between 70 and 80 per cent. The statistics of Flexner show a mortality hardly exceeding 30 per cent. in unselected cases. It must be remembered in considering the benefits of this serum that in unselected cases there must be many in which the disease has produced marked anatomical changes in the central nervous system before the serum is used. It is well known, of course, that the later manifestations of this disease, which often lead to death with hydrocephalus, asthenia, and malnutrition, are the remote results of the

[57] Flexner. *Jour. Exp. Med.*, Vol. 9, 1907, and *J. A. M. A.*, Vol. 47, 1906, p. 560.

[58] Flexner and Jobling. *Jour. Exp. Med.*, Vol. 10, 1908.

[59] Flexner. *Jour. Exp. Med.*, Vol. 17, 1913.

anatomical injuries produced by the inflammatory reactions accompanying the earlier manifestations of the acute infection. These conditions of course cannot be expected to yield to serum treatment. It must be assumed, therefore, that were we able to obtain statistics of cases diagnosed and treated soon after the onset the figures would be even more favorable than those stated above.

The following mortality table is taken from a bulletin of the Rockefeller Institute published in 1917:

Comparative Mortality Reported by Various Observers [60]

Treatment Begun	Flexner, Per cent.	Netter, Per cent.	Dopter, Per cent.	Christo-manos Per cent.	Levy, Per cent.	Flack, Per Cent.
Before third day	18.1	7.1	8.2	13.0	13.2	9.09
From fourth to seventh day	27.2	11.1	14.4	25.9	20.4	....
After seventh day	36.5	23.5	24.1	47.0	28.6	50.00

From the above table, there seems to be very little doubt that the use of the serum has very materially modified the course of the disease, and that serum treatment in meningitis is one of our most important specific therapeutic agents.

In view of the fact that we now know that many different types of meningococci exist, serological procedures must be governed by this knowledge. Typing of meningococci in different countries has led to various classifications. The first knowledge concerning the existence of various types resulted from the investigations of Dopter.[61] Dopter found that some of the meningococci isolated from cases in and about Paris did not agglutinate in the ordinary so-called normal type serum. This organism he calls the *parameningococcus,* and its discovery marks the beginning of our serological classification of these organisms. Wollstein [62] confirmed Dopter's work, and showed that in addition to the parameningococcus and the so-called normal strains, there were a considerable number of other variants that could not be classified definitely with either of the other two. During the war, Gordon,[63] in England, studied many strains of meningococci obtained from the epidemic which occurred among Canadian and British troops, and divided meningococci into four types, now commonly spoken of as the "Gordon types." Not all organisms could be classified definitely into one or the other type, but Tulloch [64] found that of 356 organisms he investigated, 234 agglutinated or gave specific agglutinin absorption in one of the four type sera produced by Gordon. Gordon's Type I corresponds to Dopter's parameningococcus. His Type II corresponds to the normal or origi-

[60] Flexner. *Bull. Rock. Inst. for Med. Res.,* 1917.
[61] Dopter. *Compt. Rend. de l. Soc. Biol.,* Vol. 67, 1909, p. 74.
[62] Wollstein. *Jour. Exp. Med.,* Vol. 20, 1914.
[63] Gordon. *Brit. Med. Res. Com. Rep.,* London, 1915-1917.
[64] Tulloch. *Jour. Roy. Med. Coll.,* February, 1918, p. 9.

nal meningococcus, and his Types III and IV represent intermediate or irregular strains of which there are a large number that shade into one another. In this country we speak of the normal, the para, and intermediate strains. In France three types have been particularly worked with, corresponding to the normal, para, and intermediate groups, and spoken of as Types A, B, and C.

While it is, of course, possible to treat cases with type serum when cultures can be obtained and type determined with sufficient speed, yet the necessity for rapid treatment makes it imperative that we work chiefly with polyvalent serum, giving the first few injections with such serum, and subsequently, as cultures are obtained, determining whether the polyvalent serum that is being used includes the particular meningococcus from which the patient is suffering .

In America and most countries, at the present time, polyvalent serum is almost entirely used. Horses are immunized with cultures of as many different types as possible. Both the normal and the paratypes, together with as many intermediates as can be collected from different epidemics throughout the country, must be used, and it is this factor, namely, the use of many different races of meningococci, which is perhaps the most important one in successful serum production. In laboratories in which serum production is being carried on, a constant survey of cases of meningitis throughout the country should be made, organisms obtained from new cases and tested against the serum. If cultures are received which do not agglutinate in the polyvalent serum, they should be added to the immunizing collection. Much judgment is necessary in this particular respect because the use of too large a number of strains may keep down the potency of the serum for any particular one.

Cultures for injection are grown upon agar and are best washed once in salt solution before injection. Usually two or three injections are made on consecutive days, and a rest of 7 to 8 days between treatments is given.

The standardization of the serum was first attempted by Flexner and Jobling on the basis of opsonin contents. In some laboratories, complement fixation has been recommended, but the usual method employed at the present time is agglutination. The serum should agglutinate normal and para types in from 1-1,000 or 1-2,000 dilutions, and should react with the intermediate strains in dilutions of 1-200 or more.

Administration of the Serum.—As we have stated above, the most important factor for success is early diagnosis and immediate treatment with serum. For this reason, responsibility of the physician in properly appraising the case, having his suspicions of epidemic meningitis aroused early, and doing a lumbar puncture, is a grave one. Delaying several days may materially influence the outcome, as may be seen from the table given above. Fluid from the

lumbar puncture should be taken into a sterile centrifuge tube and immediately sent to the laboratory for diagnosis. If the fluid which runs from the needle is turbid, and an immediate Gram stain shows Gram negative micrococci, serum should be administered at once. Moreover, we believe that if such a fluid shows a large number of polymorphonuclear leukocytes and no Gram positive organisms can be found on staining, even in the absence of Gram negative micrococci, the chances are in favor of epidemic meningitis since the meningococci undergo rapid autolysis in the spinal fluid, and in some cases it may take a long search before intact micrococci can be found. We would, therefore, be in favor of injecting serum even in such cases if an "acute" exudate is found as shown by the polymorphonuclear leukocytes and no Gram positive organisms can be seen in smears.

If serum is to be injected, spinal fluid should first be taken until the flow slows down to a drop every ten or twenty seconds. The serum is then injected through the same needle, slowly, by gravity, or if this is impossible, very slowly with a syringe. The serum should be at body temperature, and should be injected so slowly that the entire amount shall enter the spinal canal in the course of about ten minutes.

It is of the greatest importance that both in withdrawing fluid and in injecting serum, the patient should be very carefully watched for symptoms resulting from the rapid changes in intraspinous pressure.

The amount injected must to some extent depend upon the amount of fluid withdrawn, and should be a few cubic centimeters less than the amount taken out. In general, from 25 to 35 c. c. can be injected into an adult, but more may be given if a great deal of fluid has been withdrawn. Sophian recommends a simultaneous taking of the blood pressure and interruption of the procedure if the blood pressure drops suddenly.

Serum injections should be repeated as often as indicated until the fluid becomes clear and this repetition must be controlled by clinical observation, and the appearance of the spinal fluid, the number of pus cells, the number of organisms, etc.

Since it has recently been shown by Herrick [65] and a number of British observers, that in many cases of meningitis the organisms are in the blood stream even before the meningeal symptoms appear, it is advisable to inject 30 c. c. of the serum intravenously at the time at which the first intraspinous injection is made.

Moreover, it is important to remember that during epidemics, there are many cases of meningococcus septicæmia, usually accompanied by septic temperature, and in which there is often a profuse rash consisting both of red blotches on the skin and petechial spots

[65] Herrick. *Arch. Inter. Med.,* Vol. 21, 1918, p. 541.

not unlike those in typhus fever. Such cases can be diagnosed by blood culture and should be treated by energetic intravenous serum injections.

Serum Treatment in Streptococcus Infections

The attempts to produce powerful immune sera against streptococci date back to the earliest days of immunology. That the subject is a particularly difficult one follows from the great confusion which has prevailed, and, to a great extent, still prevails, regarding the classification of the streptococci and their interrelationship. There are apparently a large number of different strains of streptococci which vary from each other, not only culturally, but also in regard to agglutination and bactericidal reactions. For this reason it is not at all a foregone conclusion that a serum prepared by the immunization of an animal with a streptococcus of one type will have any protective action against other strains. In all cases in which streptococcus immune serum is at all used it must be remembered that the disease produced in human beings by organisms classified among the streptococci are by no means necessarily closely related in biological reactions, and the same immune serum may be extremely potent in one case and entirely useless in another.

It has long been known that the hemolytic streptococci which can infect man are of many different antigenic varieties, and the recent work of Dochez, Avery and Lancefield [66] has indicated that many of these organisms can be divided into at least four groups. According to the earlier work of von Pirquet, Dochez and his associates, and Tunnicliff,[67] the streptococci which are associated with cases of scarlet fever seem to constitute a separate type. However, in recent cultural studies, Rice has found marked cultural differences between organisms from this source. This question is still an open one, though the mass of evidence seems to point to a general serological similarity between the scarlatinal hemolytic streptococci. There are, nevertheless, a large number of hemolytic organisms which cannot be grouped together, and in which serological reactions seem to shade one into the other. In the viridans groups, this heterologous condition is still more marked, and studies such as those of Kinsella and Swift [68] show that these organisms are as varied as are the Type IV pneumococci. Thus, the production of therapeutic streptococcus serum is rendered extremely difficult, and even the hope of obtaining a widely useful polyvalent serum must await a more exact definition of the grouping of these organisms.

That animals could be successfully immunized against strepto-

[66] Dochez, Avery and Lancefield. *Jour. Exp. Med.*, Vol. 30, 1919, p. 179.
[67] Tunnicliff. *Jour. A. M. A.*, Vol. 75, 1920, p. 1339.
[68] Kinsella and Swift. *Jour. Exp. Med.*, Vol. 28, 1918, p. 877.

cocci was shown early in the history of investigations in immunity by a number of workers, notably Roger, Behring, von Lingelsheim, and Mironoff. The first extensive attempts to produce a curative serum for use in passively immunizing human beings were made by Marmorek [69] at the Pasteur Institute in 1895. The basic idea from which Marmorek worked was the similarity of all the streptococci producing disease in human beings. He also believed that the most powerful serum could be produced with cultures whose virulence had been greatly enhanced by animal passages. When such cultures were grown on mixtures of human serum and broth he asserted furthermore that soluble poisons were produced which could be obtained by filtration of the culture fluids. For these reasons he immunized horses with cultures rendered highly virulent by very gradual injections first of dead then of living organisms, finally injecting also considerable quantities of culture filtrates.

Testing these sera upon animals, he was successful in protecting against streptococcus infection when the serum was administered 12 to 18 hours before the bacteria were injected. He expressed the opinion that the serum was antitoxic as well as antibacterial. In his earliest reports the results of the treatment of 413 cases of erysipelas leave one very much in doubt as to the value of the serum since the difference in mortality between the treated and the untreated cases is less than 2 per cent. However, an analysis of the individual cases makes the serum treatment appear more favorable. He reported good results also in 7 cases of puerperal septicemia and in scarlatinal angina. Later observers, notably Lenhartz,[70] Baginsky,[71] and many others, have not been able to confirm the favorable results reported by Marmorek, and it may be stated that at the present day the value of Marmorek's serum is very much in question. Antistreptococcus sera have also been produced by Aronson [72] and Tavel, Van de Velde, Menzer,[73] Moser,[74] and some others. Aronson at first worked from the idea which Marmorek also had used that there was a close relationship between the various streptococci pathogenic for man. He adopted the opinion first developed by Denys [75] and Van de Velde that many different strains should be used for immunization in order to allow for possible difference in the characteristics of the pathogenic streptococci. This principle of the necessity for the production of polyvalent sera was also emphasized strongly by

[69] Marmorek. *Ann. de l'Inst. Past.*, Vol. 9, 1895.
[70] Lenhartz. "Die Septischen Erkrankungen Hölder," Wien, 1903.
[71] Baginsky. *Berl. klin. Woch.*, 1896, p. 340.
[72] Aronson. *Berl. klin. Woch.*, Vol. 39, 1902; *Deutsche med. Woch.*, Vol. 29, 1903.
[73] Menzer. *Berl. klin. Woch.*, 1902, and *Münch. med. Woch.*, 1903.
[74] Moser. *Wien. klin. Woch.*, 1902.
[75] Denys. *Bull. de l'Acad. Belge*, 1896, cited from Schwoner K. and L. H., Vol. 2.

Tavel, who based his opinion on careful agglutination tests, and by Menzer and Moser.

That the action of the antistreptococcus sera, however produced, is very largely due to its opsonic properties has been shown by Bordet,[76] by Meier and Michaelis, and a number of other workers. If there is any bactericidal power it is probably relatively slight.

It would be quite impossible to attempt in this place to analyze the large number of streptococcus infections of man which have been treated with one or the other antistreptococcus sera. Those mentioned, moreover, do not by any means include all the sera which have been produced and marketed for this purpose. In general we may say that beneficial results have been obtained chiefly in cases in which the streptococcus infection has been localized and treated early after its inception. In generalized or advanced cases it cannot be said that the results are encouraging. Even in animals, in which experimental conditions can be so much more thoroughly controlled, the protective action of even the strongest sera is evident only if the serum is administered either before infection or within a very definite period after inoculation. The standardization of streptococcus sera may be accomplished by determining its protective value for animals when injected 18 to 20 hours before infection.

Serum Treatment in Pneumonia

Attempts to work out a therapeutically valid method of passive immunization in pneumonia have been many and date from the very beginning of the discovery that pneumonia was a bacterial infection. Sera have even been marketed and used, but until recently no very encouraging results were obtained. Recent studies have revealed that in pneumonia the serum of convalescents contains practically no bactericidal properties for the pneumococcus, and that the protective powers of such serum depend upon the presence of immune opsonins or bacteriotropins, by means of which the pneumococci are rendered amenable to phagocytosis. Virulent pneumococci are not as a rule phagocytable in the presence of normal serum. However, in the presence of immune serum powerful phagocytic action can be observed. That the agglutinating action of such sera may also play an important rôle in their protective action has recently been shown by Bull (*vide infra*).

Neufeld has studied the conditions of pneumococcus immunity most thoroughly. The most important advance from a practical point of view was a discovery made by him, with Händel,[77] in 1909. They determined that there was a definite difference between various

[76] Bordet. *Ann. de l'Inst. Past.*, 1897.

[77] Neufeld and Händel. *Zeitschr. f. Imm.*, Vol. 3, 1909, and *Arb. a. d. kais. Gesundh. Amt.*, Vol. 34, 1910.

pneumococci in their reactions to immune serum; in other words, pneumococci could be grouped into various serological types. The serum produced with organisms of one type did not protect against infection with other strains. In consequence they called attention to the importance of determining the type of pneumococcus which causes the individual pneumonia so that the corresponding immune serum might be used. They produced a highly potent anti-pneumococcus serum by the injection of horses and donkeys with virulent pneumococi grown on fluid cultures, then determined the high protective power of this serum upon animals and used it in the treatment of patients by intravenous injection. Their results were exceedingly encouraging. In reporting their results Neufeld and Händel stated that considerable doses must be given. They called attention to the fact, revealed by their animal experiments, that moderate amounts do not, as in the case of diphtheria serum, exert a correspondingly slight amount of beneficial action, but that in the case of the pneumonia serum amounts smaller than a certain active minimum seem to exert absolutely no beneficial action. This is a fact which later was also determined by Dochez.

The entire subject of pneumococcus serum treatment has been further cleared by the work of Dochez and Gillespie [78] and Dochez and Avery [79] in their studies on pneumococcus types. Taking their departure from the observations of Neufeld and his associates, they have determined for the eastern United States the prevalence of four pneumococcus types which are too familiar to bacteriologists at the present time to necessitate reiteration in this place. We may, however, remind the reader that their classification divides pneumococci into two fixed types, I and II, a third group, Pneumococcus Mucosus, and a fourth heterologous group generally spoken of as Type IV. These types have also been found in Europe, and Lister has found similar typing among cases in South Africa with, however, the difference that his Type C and B corresponded to Type I and II of the American classification, but his homologous Type A has so far not been found in other countries.

The discovery of individual serologically homologous types has made possible the logical development of serum therapy. Cole, with his associates at the Rockefeller Hospital, has given the matter serious study, and has carried on extensive therapeutic investigations on a considerable number of cases. Sera produced according to his method by various health departments and commercial laboratories, have further permitted an extensive plan of investigation.

The production of the sera at the present time consists in the injection of horses with, at first, killed cultures and then living pneu-

[78] Dochez and Gillespie. *Jour. A. M. A.*, Vol. 61, 1913, p. 727.
[79] Dochez and Avery. *Jour. Exp. Med.*, Vol. 26, 1917, p. 477. Avery, Chickering, Cole and Dochez, Monograph No. 7, *Rock. Inst.*, October, 1917.

mococci. The organisms are grown on broth and young cultures are used for injection. Cole advises injecting daily for 6 days, using the sediment thrown down from 50 c. c. of a 12 hour broth culture killed at 56°. After six injections, a rest of a week is given, and the serum of the horse tested for agglutinins. This is followed by a second series of dead cultures, and again an interval is allowed. When the agglutination test works out specifically in dilutions of 1-200, and 0.2 c. c. protects against 0.1 c. c. of a highly virulent culture, the serum can be used, but usually immunization is continued with living cultures to a higher point.

The standardization of the serum is carried out by protection experiments in mice, with the determination of the amount of serum necessary to protect a 20 gram mouse against a standard virulent culture. The most important part of this technique is to possess a culture of high and constant virulence. Such cultures are produced by passage through mice. The virulence should be so great that a millionth of an 18 hour broth culture will kill a mouse in 48 hours. Dilutions are then prepared in such a way that 0.5 c. c. of a dilution contains various amounts of the pneumococcus culture, ranging from 0.2 c. c. to 0.000001 c. c. The dilutions must be freshly made in order that the number of organisms in the tubes should not materially change by growth before the test is made. With each of these dilutions, then 0.2 c. c. of the serum to be tested is mixed in a syringe and the mixture immediately injected intraperitoneally. The rule laid down by Cole is that only such sera should be employed in which 0.2 c. c. protects against 0.1 c. c. of the culture described above.

Since type serum is used, it goes without saying that the serum should be used only in cases in which the type infecting the patient is known. This must be done by obtaining sputum from a patient, taking particular care to make sure that the sputum is collected after thoroughly rinsing the mouth with bicarbonate of soda or salt solution. This is collected in a sterile Petri dish and is immediately sent to the laboratory. It is stained by Gram, and a capsule stain is made. It is then washed, dipping into successive Petri dishes containing salt solution to remove the bacteria sticking to the outside, and a small bit of the sputum is emulsified in salt solution and injected intraperitoneally into a mouse. When the mouse is either dying or dead, it should be immediately autopsied, cultures taken from the heart's blood and the peritoneal exudate, washed out with salt solution into a centrifuge tube by means of a nipple pipette. It is centrifuged for a few moments at low speed to throw down leukocytes and the turbid supernatant fluid, which should have the maximum turbidity of a well grown broth culture, is transferred to another centrifuge tube and centrifuged at high speed to throw down the organisms. This sediment is resuspended and tested with type sera for agglutination according to the following scheme taken

directly from the Monograph by Avery, Chickering, Cole and Dochez.[80]

Other methods of typing have been devised for exceptional cases which can be found described in any of the more recent textbooks of bacteriology.[81]

Determination of Pneumococcus Types by Agglutination

Pneumococcus Suspension, 0.5 c. c.	Serum I (1:20) 0.5 c. c.	Serum II (undiluted) 0.5 c. c.	Serum II (1:20) 0.5 c. c.	Serum III (1:5) 0.5 c.c.
Type I	++	..	..	..
Type II	..	++	++	..
Sub-groups 11a, b, x......	..	+	..	..
Type III	..	..	..	++
Type IV	..	..	..	..

Incubation for 1 hour at 37° C.

Although much has been written about the results of serum treatment in pneumonia, we can for the present do no better than accept the judgment of Cole and his co-workers who have studied the problem extensively and have, in various places, published an unprejudiced opinion. For some unknown reason, no success has been obtained with anything but Type I serum. For the present, therefore, there is no experimental basis for serum treatment in cases other than those caused by the Type I pneumococcus. According to Cole, there seems to be very little doubt that in Type I cases the use of serum has reduced the mortality by about 50 per cent., and while there are many dissenting opinions, this, as far as we can ascertain, is the general judgment of most clinicians with whom we have discussed the matter, who have given the method serious test.

We can assume, therefore, that in Type I pneumonias, the use of serum is indicated. It is, of course, necessary to use it as early as possible in the disease. Skin reactions for horse serum hypersensitiveness should be done on the patient and, if necessary, desensitization should be undertaken, according to the principles described in another section of this book.

For first injection, Cole [82] advises 90 to 100 c. c. of the serum, diluted 50 per cent. in salt solution and injected intravenously by gravity with such slowness that the first 10 c. c. take about 10 to 15 minutes to run in. Cole establishes the effectiveness of the serum by the fact that the patient's blood becomes sterile, that often the progression of local lesions in the lung is arrested, and that there is a

[80] Avery, Chickering, Cole and Dochez. *Loc. cit.*

[81] See Hiss, Zinsser and Russell. "Textbook of Bacteriology," 5th Edition, D. Appleton, New York, 1922.

[82] Cole. *Jour. A. M. A.*, Vol. 76, 1921, p. 111.

great amelioration in objective and subjective symptoms. He states that the mortality rate in untreated lobar pneumonias of Type I has been shown to be from 25 to 30 per cent. Of 495 cases collected, the mortality was 10.5 per cent. after serum treatment. Further statistical studies will settle this question.

In addition to the reactions generally classified as anaphylactic following the injections of the serum, serum sickness may occur, and there may be a marked thermal reaction within 20 minutes or one hour, following administration. This is probably similar to that described in another section of this book in connection with the injection of non-specific protein and protein cleavage products in various infections.

The exact manner of action of the serum is, of course, unclear. Dochez has shown that in untreated pneumonia cases, antibodies do not appear in the serum until about the time of the crisis. In the treated cases, of course, antibodies are supplied immediately after the injection of the serum and, according to the studies of Bull detailed in another part of this book, an almost immediate agglutination of the pneumococci in the blood stream takes place. If nothing else is accomplished by the serum injection, the reduction of the septicemia may be of enormous benefit to the patient.

More recently the treatment of pneumonias with the so-called purified antibody extracts of Huntoon has been extensively attempted by Cecil and his co-workers in New York. These antibody preparations have been described in detail in our section on the dissociation of antigen from antibody.

The Huntoon pneumococcus antibody extracts have been used particularly by Cecil and Larsen [82a] at Bellevue Hospital in New York, and by Dr. Lewis A. Conner.[82b] Both observers carefully typed their cases in every instance. The antibody extracts were made with horse serum potent against Types I, II and III, as a point of departure. This serum was absorbed with heavy emulsions of the organisms of these types until the suspensions were heavily agglutinated, the sediment washed with salt solution, and finally extracted in 0.25 per cent. sodium bicarbonate at 55° C. for from three-quarters to one hour. The material was prepared by Huntoon, contained protective substances against Types I, II and III, equal in amount to potent polyvalent antipneumococcus serum. The conclusions of Cecil and Larsen from a considerable number of cases are as follows: In 424 cases of pneumococcus pneumonia treated with the antibody solutions, the death rate was 21.4 per cent., while in a control series of 428 cases in the same institution, the death rate was 28.3 per cent. The most striking results of the pneumococcus antibody were observed with Type I. In 156 treated Type I cases, the death rate was

[82a] Cecil and Larsen. *Jour. A. M. A.*, 79, 1922, p. 343.
[82b] Conner, Lewis A. *Amer. Jour. Med. Scien.*, 164, 1922, p. 832.

13.3 per cent., while a control series of 162 showed a death rate of 22.2 per cent. A definite but less marked effect was seen in the Type II and Type IV cases. There was no effect whatever in Type III cases. Streptococcus pneumonias were not favorably influenced. There seemed to be a shortening of the course of the disease also, in that 28.8 per cent. of the treated cases recovered on or before the fifth day, while only 7.9 per cent. of the untreated cases recovered as early as this. There was a slight difference in the severe complications occurring in treated and untreated cases, in favor of the treated cases. These statistics, gathered from a very carefully observed material, seem definitely to show that a certain amount of therapeutic value may be attributed to the antibody extracts, but it may still be questioned whether with such relatively small differences, this should be interpreted as a specific immunological protection, or perhaps as the non-specific effect of the bacterial materials injected with these extracts. Moreover, it does not permit us to compare the relative values of antibody extracts and pneumococcus serum.

Similar studies by Lewis A. Conner, again in very carefully typed cases, are summarized by him as follows: In Conner's observations there were no untreated controls used, partly because of the relatively small number of cases of this kind coming to the hospital in the course of a year, and probably also because of a conscientious feeling that if the treatment proved effective, it would be unfair not to let every patient have the benefit of it. His observations were carried over two winter seasons in order to lessen the possibility of seasonal variations in the severity of pneumonias and consequent mortality; 116 cases of lobar pneumonias in adults were treated during this period, with a death rate of 14.6 per cent. Among these were 13 Friedlander and streptococcus pneumonias, which showed a death rate of 46.1 per cent. He calls attention to the fact that 54.4 per cent. of the cases happened to be Type IV, with the very low mortality of 4.1 per cent., and he justly concludes that since the effect on the Type IV cases seemed to be as potent as the effect on Types I and II, one might naturally question the specific nature of the benefit derived from it. That the non-specific element is a strong one, would also follow from the relatively severe immediate reactions following the injections. In one patient, Conner says that he believes death may have been directly traceable to the severity of this immediate reaction. He, nevertheless, believes the method to represent a forward step in rational treatment of pneumonia.

The Serum Treatment of Typhoid Fever

The first extensive attempts to treat typhoid fever by passive immunization with the serum of treated animals were made by Chantemesse, who immunized horses with filtrates of typhoid cultures sub-

cutaneously and with emulsions of virulent bacilli intravenously. Chantemesse believed that the serum of horses which had been treated in this way for very long periods possessed, not only bactericidal action, but stimulated phagocytosis, and possessed a certain limited amount of neutralizing power against the toxic properties of the typhoid filtrates. At the International Congress of Hygiene in Berlin in 1907 Chantemesse [83] reported upon a thousand cases treated with his serum. Of this number 43 only died, whereas the average mortality during the same six years at the Paris hospitals was 17 per cent. The injection of the serum he claimed very markedly improved the condition of patients in that, after a preliminary period of no apparent change lasting from several hours to 5 or 6 days, the temperature goes down and the general condition of the patient changes considerably for the better. He noticed very few complications in these cases, and intestinal hemorrhage occurred four times only.

A remarkable feature of Chantemesse's treatment is that he injected into the patients a few drops only of the serum, and rarely made a second injection, facts which alone tend to persuade one that his apparent therapeutic success was a fortunate accident.

The opinion originally expressed by Chantemesse that the serum of horses vigorously treated with typhoid bacilli possesses in addition to its bactericidal and opsonic powers definite antitoxic properties recurs again in the work of a number of investigators. Besredka [84] prepared a serum by the intravenous injection of typhoid cultures heated to 60° C., continuing the immunization for 6 months. He claims that this serum possesses what he designates as "anti-endotoxic" properties. A dry extract of typhoid bacilli which in dose of 0.01 gram killed a guinea pig of 300 grams regularly became innocuous when mixed with small quantities of this horse serum. One c. c. of the horse serum neutralized often as much as two fatal doses of the serum, but it is important theoretically to recognize that Besredka states particularly that even an increase of the quantity of serum never neutralized more than two fatal doses. This is particularly important in connection with the more recent studies on toxic split proteins by Vaughan, and on anaphylatoxins by Bessau and by Zinsser and Dwyer, in which it has been shown that an animal acquires a tolerance against the toxic substances produced from bacterial and other proteins which, however, never exceeds one or two multiples of the minimum lethal dose. This fact alone would militate against considering the serum of Besredka in any way antitoxic in the sense in which the word is used concerning diphtheria and tetanus antitoxins where neutralization of poison follows roughly the law of

[83] Chantemesse. International Congress of Hygiene, Berlin, September, 1907; Ref. *Bull. de l'Inst. Past.*, Vol. 5, 1907, p. 931.

[84] Besredka. *Ann. de l'Inst. Past.*, Vol. 19, 1905, and Vol. 20, 1906.

multiples. Besredka's anti-endotoxic sera has recently been very thoroughly investigated by Pfeiffer and Bessau.[85] These investigators have found that Besredka's serum exerted a very definite beneficial influence upon typhoid infection in guinea pigs if injected at the same time with the bacilli. In their experiments it also protected somewhat against the toxic properties of substances derived from the typhoid bacillus, and Pfeiffer and Bessau did not believe that this was due to a true antitoxic action, nor that the serum was superior in this respect to the ordinary bactericidal sera prepared by inoculating animals with typhoid bacilli. Kraus and Stenitzer[86] have also taken up the study of typhoid immunization from the point of view that the typhoid bacillus produces a true toxin, and that therefore a true antitoxic action could be expected from the sera produced by immunization with typhoid filtrates. It should be noted that, in spite of the most common opinions against this at present, a similar point of view was advanced by MacFadyen,[87] and more recently by Arima.[88] Kraus and Stenitzer[89] immunized horses and goats very highly with extracts of agar cultures and with broth filtrates by intravenous injection. The serum which they produced in this way not only possessed the ordinary bactericidal action, but, they claimed, neutralized also toxic broth filtrates, not only of the typhoid, but of the paratyphoid bacilli. The serum of Kraus and Stenitzer has been used by a number of observers, among whom are Herz,[90] Forssmann, Unger, Russ, and others, and the results are said to be encouraging in early cases.

Rodet and Lagrifoul[91] immunized horses with living typhoid cultures, and also claim favorable results.

Mathes,[92] continuing the work of Gottstein after the death of the latter, employed the method of immunizing with the product obtained by digesting typhoid bacilli with trypsin. The poison so produced he speaks of as "fermotoxin." Lüdke[93] slightly modified the Gottstein-Mathes method by digesting the typhoid bacilli with pepsin and hydrochloric acid, and with the poison so produced immunized 8 goats, reënforcing the immunization by the subsequent injection of the bacilli themselves. He claims that 0.05 to 0.1 c. c. of the serum so produced protected animals against five times the lethal dose

[85] Pfeiffer and Bessau. *Centralbl. f. Bakt.*, Vol. 56, 1910.

[86] Kraus and Stenitzer. *Wien. klin. Woch.*, Vol. 20, 1907, pp. 344, 753, and Vol. 21, 1908, p. 645.

[87] MacFadyen. Cited from Stenitzer in "Kraus und Levaditi Handbuch," Vol. 2.

[88] Arima. *Centralbl. f. Bakt.*, Vol. 65, 1912, p. 183. Orig.

[89] Kraus and Stenitzer. *Wein. klin. Woch.*, Vol. 22, 1909, p. 1395; *Deutsche med. Woch.*, March, 1911.

[90] Herz. *Wien. klin. Woch.*, Vol. 22, 1909, p. 1746.

[91] Rodet and Lagrifoul. *C. R. de la Soc. de Biol.*, April, 1910.

[92] Mathes. *D. Archiv f. klin. Med.*, Vol. 95, 1909.

[93] Lüdke. *D. Archiv. f. klin. Med.*, Vol. 98, 1910.

of the poison. In a small series of human cases treated by this method he reports good results.

Garbat and Meyer [94] immunized animals with sensitized typhoid bacilli, and claim that the most potent sera for typhoid immunization can be obtained by the combination of sera produced by the injection of sensitized and of unsensitized bacteria. They assert that the typhoid bacillus contains two definite antigens, one particularly associated with the bacterial ectoplasm, which becomes active when the bacteria enter the animal body, and a truly endocellular poison which does not become active until the surrounding ectoplasm is dissolved. They believe that sensitizing bacteria is a method for the production of endotoxin, and think that therefore the ideal serum for the treatment of typhoid consists of a mixture of two sera produced each with one of the antigens, that is, with sensitized and unsensitized bacteria. Rommel and Herman [95] did not obtain encouraging results with this serum.

From a study of the literature it seems to us that in spite of the many different methods of production employed by various observers in their studies on typhoid sera it is quite likely that all these sera are essentially alike, containing, quantitatively, according to the degree of immunization, bactericidal, agglutinating, and opsonic properties, with possibly a limited amount of neutralizing power for the poisons liberated from the typhoid bacilli in the body. As far as we can judge from clinical reports the therapeutic value of the sera so far produced is not very great. It seems that cases treated early in the disease may be benefited, and possibly an early cessation of the bacteriemia can in this way be attained. However, it does not seem either theoretically or from the study of clinical publications that any very marked effects have followed the use of any of the sera in advanced cases.

The Serum Treatment of Plague

That the serum of animals immunized with killed plague cultures may actively protect normal animals from experimental infection was first shown by Yersin, Calmette, and Borrel.[96] The serum which they produced possessed apparently powerful bactericidal action, but no antitoxic properties were demonstrated. They determined its protective powers by injecting measured quantities into mice and infecting them with fatal doses of virulent plague bacilli 24 hours later. The Yersin serum which was produced for the treatment of plague as a result of these experiments was made, then, by the gradual immunization of horses with first dead plague bacilli, finally with virulent living organisms. The serum has been extensively

[94] Garbat and Meyer. *Zeitschr. f. exp. Path. u. Ther.*, Vol. 8, 1911.
[95] Rommel and Herman. *Centralbl. f. Bakt.* Ref. Vol. 53, 1912.
[96] Yersin, Calmette, and Borrel. *Ann. de l'Inst.* Past., 1895.

used by many observers with results that leave one much in doubt as to its efficacy. Yersin [97] himself, reporting on an epidemic in Nhatrang, reports a general mortality of 73 per cent. for the whole epidemic, a mortality of 100 per cent. in untreated cases, and of 42 per cent. among those treated with his serum. Good results were also reported from the epidemics in Amoy and Canton in 1896. However, these results apparently were not accepted by all observers as proving the efficiency of the serum, since the number of cases observed were few, and the irregularity in the gravity of the disease in different individuals makes statistical evidence unreliable unless large material can be studied. Kolle and Martini [98] announce that Dr. Choksy reported very poor success with the Yersin serum, and cite a number of later writers whose results with this serum were also unsatisfactory when used on human beings. That the serum unquestionably contains antibodies against the plague bacillus is testified to, not only by the French observers themselves, but also by the German Plague Commission of 1899, and by Kolle and Martini [99] themselves. The Commission experimented with this serum upon monkeys, and showed that it possessed unquestionable protective powers in rodents and in monkeys when given 24 hours before the plague infection, and in monkeys possessed fair curative properties when injected 24 hours later than inoculation with the plague bacilli. Because of the doubtful success in the treatment of human beings with this serum Yersin and Roux at the Pasteur Institute later altered their methods of serum production by injecting, not only dead and living plague cultures, but considerable quantities of culture filtrates after the horses had attained a high degree of immunity. Later observations on the Yersin [100] serum have been published by the British Plague Commission in 1908 and 1911. In this investigation the cases were controlled as to their severity by blood culture, since it had been claimed by a number of earlier investigators that the Yersin serum was efficient in mild cases, but failed entirely in the severe ones. It seems from the report of this Commission that ordinarily 70 per cent. of cases of plague without bacilli in the blood survive while three-quarters of those with mild septicemia die, and all of those with a marked septicemia succumb. In the summary given of 146 cases treated with Yersin's serum by the British Commission 65.1 per cent. died, whereas of 146 untreated controls 71.90 per cent. died. These fixures, together with an analysis of the percentages, classified according to the severity of the infections, do not show a very marked curative action on the part of the serum.

[97] Yersin. *Ann. de l'Inst. Past.*, 1899.
[98] Kolle and Martini. *Deutsche med. Woch.*, 1902, p. 29.
[99] German Plague Commission. *Arb. a. d. kais. Amt.*, Vol. 16, 1899.
[100] British Plague Commission. *Jour. Hyg.*, Vol. 12, Sup., 1912, p. 326.

Markl,[101] who claims that the plague bacillus produces a soluble toxin, has produced a plague serum by immunization of animals by filtrates of broth cultures. He claims that 0.1 c. c. of his serum, as produced at Vienna, will protect various animals against lethal doses of plague bacilli if given at the same time. He attributes much of its curative action to the fact that in the presence of this serum active phagocytosis takes place.

Dean,[102] in 1906, also claimed to have produced strongly antitoxic plague sera by treating horses with filtrates from 8 to 10 weeks' old bouillon cultures. He claims that 1 c. c. of his serum will neutralize 150 or 450 minimal lethal doses of the plague poison according to whether one measures the M L D by death in 48 hours or in 4 days. Rowland [103] also has produced a serum by the immunization of animals with the "toxins" produced by his sulphate process. Rowland has apparently utilized the idea previously advanced by Lustig of immunizing with "nucleoproteins" derived from the plague bacillus instead of with the whole bacteria. Lustig's [104] method consisted of washing up agar cultures of plague bacilli in 1 per cent. sodium hydrate solution, precipitating with ascitic acid, taking up the precipitate in an indifferent fluid and injecting it into horses. The serum produced by Lustig's method was used in Bombay, and is reported by Hahn as effective in milder cases, but without action in the more severe ones. There was but slight difference in the latter type between the treated and the untreated cases.

Rowland's [105] method consisted in the treatment of the moist bacteria with enough anhydrous sulphate of soda to combine with all the water present, freezing and thawing the mixture and filtering off the bacterial deposit at 37° C. Subsequently he extracted this bacterial mass with water. The extract so obtained was fatal to rats in quantities of 0.05 to 0.1 mg., killing them in 18 hours. In his experiments doses of 0.001 to 0.01 afforded protection, the last-named quantity reducing the mortality after inoculation of fatal doses from 80 per cent. to 10 per cent.

The sera produced by the immunization of horses with these supposed nucleoproteids are taken to be antitoxic in nature by Rowland himself and by MacConky. They were used in the treatment of plague cases in the epidemics of 1908 and 1911 by the Maratha Hospital in Bombay, and reported upon by the Indian Plague Commission on the basis of observations made by Dr. Choksy. The cases in

[101] Markl. *Centralbl. f. Bakt.*, Vol. 24, 1898; *Zeitschr. f. Hyg.*, Vol. 37, 1901; *Zeitschr. f. Hyg.*, Vol. 42, 1903.

[102] Dean. Cited from MacConky, *Jour. Hyg.*, Vol. 12, Plague Suppl. II, 1912, p. 402.

[103] Rowland. *Jour. Hyg.*, Vol. 11, Plague Suppl. I, pp. 11-19.

[104] Lustig. "Monograph Sierotrapia e Vaccin Prev. Control la Peste," Turin, 1899; cited from Kolle and Martini, *loc. cit.*

[105] Rowland. *Jour. Hyg.*, Vol. 10, p. 536.

this series were controlled, as were those treated by the Yersin serum, by blood culture. Here the results were not striking—68.40 per cent. of the serum-treated cases died, while 77.60 per cent. of the controls died.

Altogether we cannot draw any definite conclusions as to the value of the serum treatment in plague. On the whole it does appear that the milder cases are materially benefited by the treatment, and it is not at all impossible that in such cases aggravation of a milder case into fatal septicemia may be prevented by the timely administration of the plague serum. Animal experimentation also seems to indicate that the administration of the serum may be of great value as a prophylactic measure. It seems, on the other hand, as far as we can judge from the evidence of statistics, that when a case of plague has developed into the condition of active septicemia the administration of even the strongest plague sera at present available is of little use. And this is indeed unfortunately true of all passive immunization where the activity of the serum seems to depend chiefly upon bactericidal and opsonic properties. For we cannot definitely accept at the present day the claims that a true antitoxic serum, in the sense of those produced against diphtheria and tetanus poisons, can be really produced in the case of plague. The toxic substances derived from plague bacilli by a number of observers do not correspond in many particulars to true toxins.

Passive Immunization with Anthrax Serum.—Anthrax serum has been prepared by a great many observers, and has been more closely studied by Sobernheim.[106] The serum may be prepared by the active immunization of sheep. Beginning with a preliminary treatment with Pasteur's vaccines and following this with gradually increasing doses of eventually virulent anthrax bacilli. Sheep are preferred by Sobernheim for serum production. He believes that a potent serum has very definite beneficial effects in the passive prophylactic protection of cattle, sheep and horses, and may even save animals that have been infected. Sclavo has also applied passive immunization in infected human beings. According to him, the best method of treatment is the injection of 30 to 40 c. c. of serum subcutaneously, followed in 24 hours by a similar dose. He has injected 10 c. c. or more intravenously in severe cases. As far as we can tell from the collected cases, serum treatment is advisable in all cases in which a severe anthrax infection has been determined.

INFECTION AND IMMUNITY IN POLIOMYELITIS

The active experimental investigation of poliomyelitis started with the discovery by Landsteiner and Popper,[107] that monkeys

[106] Sobernheim. *Zeitschr. f. Hyg.*, Vol. 25, 1897, and Vol. 31, 1899.
[107] Landsteiner and Popper. *Zeitschr. f. Immunitäts.*, Vol. 11, 1909.

could be inoculated with this disease by the intracerebral or the intraperitoneal injection of saline emulsions of brain or spinal cord of individuals dead of the disease. The same observations by Flexner and Lewis [108] a little later, and then by a large number of workers throughout the world, gave an opportunity for the careful study of immunological conditions, and of the nature of the virus. It is our own opinion that the disease is caused by the globoid bodies of Noguchi and Flexner [109] and not by streptococci. However, the work on this controversy may be regarded as unfinished at the present writing, and we may abstain from a prolonged discussion of the etiological factor. The mere fact that the virus of poliomyelitis has been found to keep as long as four to six years in glycerinated nervous tissue, and the analogy which this offers to such virus as that of rabies, small-pox, etc., make it alone seem likely that true bacteria are not concerned in the cause of the disease. Our own experiments, and those of Dr. Tsen of this laboratory, on the isolation of streptococci from poliomyelitis animals, incline us to think that animals afflicted with this disease are readily subject to secondary tissue infection with organisms of various kinds, but chiefly with streptococci of the viridans type, which are so universally distributed throughout the body.

It has long been suspected that one attack of poliomyelitis protected from subsequent infection. It is plain therefore that some form of active immunization takes place in the course of the disease. Experimentation on monkeys subsequently confirmed this, in that it was shown that monkeys that had contracted the disease, and recovered, were thereafter resistant to inoculation. Strangely enough, however, monkeys that have been unsuccessfully inoculated are just as susceptible as they were before, showing that the immunity in this disease is closely analogous to that existing in syphilis and some other diseases where inoculation with attenuated virus, dead virus, or sub-infectious doses of living virus, is entirely incapable of producing immunity.

Early in the study of poliomyelitis it was found that one attack protected and that the serum of a human being or monkey that had recovered from the disease was capable of neutralizing the virus. The artificial production of immune sera by the injection of virus has been generally unsuccessful. The only hopeful fact we know in this regard is the fact that the serum of individuals who have recovered from an attack of the disease will neutralize the virus if mixed with it before injection. For this reason, Netter,[110] Netter and Salanier,[111] Amoss and Chesney [112] and others have attempted

[108] Flexner and Lewis. *Jour. Amer. Med. Assn.*, Vol. 44, 1910, p. 45.

[109] Flexner and Noguchi. *Jour. Exp. Med.*, Vol. 18, 1913, p. 461.

[110] Netter. *Bull. de l'Acad. Med.*, Series 3, Vol. 74, 1915, p. 403.

[111] Netter and Salanier. *Bull. Soc. Med. des Hop. de Paris*, Series 3, Vol. 40, 1916, p. 299.

[112] Amoss and Chesney. *Jour. Exp. Med.*, Vol. 25, 1917, p. 581.

to use the serum of recovered cases in the treatment of patients with the disease. The following summary of the treatment is taken largely from Amos.[113] Blood is withdrawn from patients that have recovered from the disease in quantities varying according to the size and age of the subject. In children of ten, as much as 200 c. c. may be taken, and in adults 500 c. c. or even more. The serum, separated from the clot, is inactivated at 55° for one hour and tested for sterility. Wassermann reactions should be done on the donors. The administration of the serum after the febrile period is, according to Amoss, of very doubtful value. It must be administered early before the onset of paralysis and, for this reason, a microscope carried to the bedside may make it possible to examine the fluid for cells and globulin while the needle is still in place, and if the diagnosis is a positive one, intraspinous injection of the serum may be given immediately through the same needle without a second puncture. Fluid is withdrawn until the flow is quite slow—drop by drop. Amoss advises slow injection, by the gravity method, of an amount of serum which equals the amount of fluid withdrawn, less ten in terms of cubic centimeters. Care should be taken that the serum is warmed to body temperature before injection. Repetition of the treatment may be practiced after 24 hours, if the temperature is not normal. No other form of serum treatment has had any effect in this disease so far.

[113] Amoss. *Jour. A. M. A.*, Vol. 76, 1921, p. 110.

CHAPTER XXI

THERAPEUTIC IMMUNIZATION (*Continued*)

Active Immunization in Man. (Prophylactic and Therapeutic.)

In discussing the value of active immunization in man we must distinguish sharply between active immunization which is prophylactic and that which is carried out after the disease has gained a definite foothold in the body. In the former case we are dealing with an old method and with one upon which the very foundations of our knowledge of immunity have been built. It is the method of Jenner in small-pox. It is that of Pasteur in chicken cholera, in anthrax, and in many other infections. It has been used as a routine in animal experimentation in laboratories since the first days of the systematic study of infections. There is no question about its being a rational and logical procedure. The immunity which can be easily conferred upon a healthy individual in this way need not be extensively above the normal in order to protect from invasion by the small numbers of pathogenic germs which may gain entrance under conditions of accidental, spontaneous infection.

The possibilities of the method were recognized by Ferran, a pupil of Pasteur, who applied it to cholera, and, since his time, it has been extensively attempted in many of the infectious diseases which occur epidemically, and therefore justify attempts in this direction. In essence also Pasteur's method of active immunization in rabies represents such prophylactic vaccination, since, in this case, although treatment is begun after infection has taken place, nevertheless the process of immunization is carried out during the incubation period before active manifestations of the disease have set in. Prophylactic vaccination, therefore, is a valuable procedure which has reaped remarkable results of recent years, especially in protection against typhoid fever. In a subsequent section this phase of vaccination is more extensively discussed, and we may therefore leave it for the present.

An entirely different problem is presented by the conditions prevailing in cases in which a bacterial infection is actually going on at the time that vaccine treatment is proposed. Here the bacteria are already present and growing in the body, and a certain amount of antibody reaction is being stimulated automatically as a result of their presence. In how far the administration of vaccines is justifi-

able or even logical in such cases, is a question which cannot be briefly answered and which depends upon an analysis of the prevailing conditions in each individual case.

We can approach the problem best by roughly classifying the various forms in which infection occurs in the human being.

When bacteria gain entrance into the tissues of the human body, granted that the organisms are pathogenic, an immediate struggle ensues between the offensive properties of the micro-organisms and the defensive powers of the tissues. The factors which determine the outcome of such a combat have been more fully considered in Chapter I. Briefly, if the defensive powers of the body greatly preponderate the result is localization and rapid destruction of the micro-organisms—with cure. In such a case any form of treatment is unnecessary. On the other hand, the balance of power may be turned in the opposite direction, in which case the infectious process becomes rapidly generalized, the bacteria enter the blood stream and lymphatics, and the defensive powers are overwhelmed. In such a case also active immunization with vaccines is entirely useless.

There are cases, however, in which the struggle is a more equal one, and in which the infectious process is held in check by the defenses, so that it takes a slow, chronic, localized form, and spreads, if at all, very slowly. What is it in such a case that prevents complete healing of the process? The answer to this may be found both in local and in systemic causes. Locally the lesion, after the preliminary skirmishes, may become encapsulated either by fibrin formation, clot, or other tissue changes so that, as Wright suggests, the fluid constituents of the blood-plasma cannot easily approach the organisms in the lesion. The same effect may result from internal pressure by fluid and possibly by the presence of considerable quantities of tissue detritus, by which protective serum constituents are fixed and thus diverted from the bacteria. Against these factors, of course, no form of immunization can be of value. Wright recognizes this, and suggests the use of surgical evacuation, Bier's method, X-rays, Finsen light, heat, and a number of other localized methods of increasing the blood supply. This, too, may be the reason for the benefits derived from wet dressings, in that they keep the tissues macerated, soft, and moist. At any rate, it is a matter of local surgical treatment. At the same time, however, there may be systemic causes which prevent the complete healing of such lesions, namely, an insufficient supply of circulating antibodies, opsonic or bactericidal substances. These may be sufficient to hold the lesion in check, but since small quantities of bacteria only are in contact with the blood stream, relatively small amounts of antigen are absorbed and antibody formation is consequently deficient. Here we have an ideal condition for vaccine therapy. By isolation of the

organisms from the patient's lesion, for which, in this case, there is time, and the careful immunization of the patient with these organisms, the immunity may be considerably increased and cure effected.

Closely related to this type of lesion are those conditions in which there are localized infections which heal rapidly but recur in quick succession again and again. Such are the common cases of consecutive crops of boils; and not dissimilar are the manifestations of erysipelas where the lesion extends along the edges while it heals in the center. There is in this type, probably, a very close balance between protection and offense; the defensive reaction is sufficient to overcome the localized lesion, but insufficient to set up a permanent systemic protection. A certain amount of local immunity acquired by the tissues of the affected areas may suffice to throw slight weight into the balance on the side of protection, enough at least to decide the struggle; and this element of locally acquired tissue resistance is in all probability also the cause for the failure of these lesions to recur immediately in the same area. Here, too, treatment with vaccines is not illogical and may yield good results if properly carried out.

In generalized systemic infections we must sharply distinguish between cases of acute sepsis in which the bacteria are actively growing and multiplying in the circulation and other cases in which blood cultures are positive only because the bacteria are being constantly discharged into the circulation from a focus in the tissues. In the former the defenses of the body are overwhelmed by an extensive flooding with the bacteria, and vaccines, if not harmful, are, at any rate, utterly useless since the antigen is already so extensively distributed throughout the tissues that if the body were capable of responding with sufficient antibody formation this would unquestionably occur without the small additional amount furnished in the bacterial emulsion. Vaccination in such cases is entirely analogous to an attempt to stimulate a degenerated heart muscle with strychnin.

Such cases of septicemia, however, are not in our opinion the most common ones in the human being. It is probable that all localized infections of more than a very trifling nature discharge living bacteria into the circulation from the very beginning. However, in most cases the bacteria, though able to hold their own in their entrenched position at the focus where accumulated offensive factors and local injury reënforce them, are yet rapidly destroyed when, in small detachments, they get into the open circulation where the plasma antibodies and phagocytes are freely active. There are cases which take a middle course between such purely localized lesions and the acute septicemia, conditions in which a well-established focus continues to furnish bacteria to the blood stream as fast as they are destroyed. An example which illustrates our meaning

well is that of the so-called subacute endocarditis caused by the *Streptococcus viridans* and its close biological kin, where blood cultures are often consistently positive for a long period or may show occasional intervals in which the blood is bacteria-free. The focus on the heart valves apparently can continue uncured in spite of a relatively high or at least normal systemic resistance to the microorganisms. If, as we ourselves have done, we isolate the organisms by blood culture from such cases, and then measure the opsonic properties of the patient's own serum against them, using the patient's own leukocytes, we may often find that active phagocytosis takes place, in a degree equal or even superior to that taking place in the serum of normal individuals. Neither does there seem to be a diminished phagocytic power of the patient's own leukocytes. For a long time these conditions may continue, with a constant destruction of bacteria in the blood and a corresponding renewal of the supply from the lesion. The same condition can be observed in rabbits in which chronic endocarditis with persistently positive blood culture has been produced by injections of these bacteria. In such animals measurements similar to those described above have been made by Miss Gilbert in our laboratory, and it has seemed as though persistently positive blood cultures could be obtained only when a localized focus was set up in the animals. Unless this is the case the blood cultures rapidly become negative.

Conditions essentially similar may exist in any other form of severe localized infection. Positive blood cultures do not necessarily mean a multiplication of the bacteria in the blood stream and a rapid overwhelming of the body. We have had occasion to see a number of cases of bacteriemia in which the focus of infection was surgically accessible; and in some of these cases early removal of the focus and purely surgical treatment resulted in a clearing up of the infection. Similar experiences have been reported by Libman and a number of others, and for this reason general septicemia, if not fulminating, may still be less desperate than ordinarily supposed.

Now, having outlined the conditions obtaining in such cases, let us briefly consider whether, under the circumstances, vaccine therapy may logically be regarded as a hopeful form of treatment. We may assume, on the one hand, that the bacteria, being consistently present and destroyed in the blood, should furnish antigen sufficient to stimulate the body tissues to their utmost reactive ability. This would seem a strong argument against vaccine therapy. On the other hand, we must take into consideration another phase of the subject, one which has some experimental justification. In discussing the origin of antibodies in another section it will be remembered that we called attention to the fact that many different tissue cells probably participate in the production of these protective reaction-bodies. We cited an experiment of Wassermann and his pupils in

which they proved that antibodies were produced most energetically in the tissues about the point of injection of the antigen, namely, in the place at which it came into most concentrated contact with the cells. They injected bacteria into the subcutaneous tissues of the ear of a rabbit, measured the progressively increasing appearance of antibodies in the blood stream, and then amputated the ear. A sudden drop of antibody contents followed, showing that the supply of antibodies had largely emanated from the tissues surrounding the injection point. Park [1] has pointed out another reason why vaccine treatment might be expected to exert beneficial action in such cases. He calls attention to the fact that when very large amounts of antitoxin are added to toxin before injection no antibody production results, and assumes that in chronic or subacute general infections the circulating bacteria are in contact with specific antibodies, partially "sensitized," and therefore not efficient as antigen. In consequence the injection of homologous unsensitized bacteria may hasten antibody formation. This assumption of Park is theoretically valid, but it is not in accord with the more recent experiments of Metchnikoff and Besredka, who claim to have obtained the best results in prophylactic typhoid vaccination by the injection of sensitized bacteria.

In acute diseases which run a definite course, typhoid fever, pneumonia, dysentery, cholera, plague, and a number of other conditions, vaccine treatment during the course of the disease has not much theoretical justification. In typhoid fever, especially, specific antibodies appear in the blood in amounts enormously increased above the normal at periods when the patient is still actively ill in spite of the fact that the blood stream has been freed of the micro-organisms. Whatever may be our opinion as to the continuance of the disease after bacteria have been driven out of the blood stream, the use of vaccines can only tend to further increase of antibodies which are already present in amounts far exceeding normal. In pneumonia the micro-organisms seem curiously resistant against the attack of the serum antibodies, and in spite of the presence of large amounts of antigen both in the lungs and, for a time, in the circulation the development of immunity is delayed until just before or near the crisis. Since this, however, is usually only a matter of 7 or 8 days, it is hardly likely that the injection of vaccines during this period could markedly alter the ultimate outcome. In a later section we shall see that vaccine treatment in typhoid fever is nevertheless being extensively tried and gives non-specific reactions of a nature which cannot entirely be explained on the basis of the above considerations.

Thus, the use of vaccines in subacute or chronic cases of infection finds much theoretical justification in all conditions in which a slug-

[1] Park. *Trans. of Americ. Phys.*, Vol. 8, 1910.

gish, localized process persists without much tendency to spread, but also without sufficient progress in the direction of cure. Theoretically, also, we may justify the attempt at vaccine treatment in subacute or chronic cases in which bacteria appear in the blood stream, constantly fed into it from a localized focus. Like so many other phases of this question, the ultimate answer lies with clinical experience, for experimentation upon animals cannot imitate entirely the conditions prevailing in man. Also, in man there are many modifying accidental factors which cannot be controlled by animal experiment. Clinical experience has not yet brought the necessary reply, and in spite of many attempts to summarize the available literature, no generalization is as yet justified. All that we can say at the present time is that active immunization with homologous vaccines is one of our most useful methods of prophylactic protection. It is extremely useful, though not universally successful in cases of often repeated localized infections, such as boils and furuncles in which there are free intervals between attacks during which an opportunity is given for artificial enhancement of the resistance. It is justified and hopeful in cases of sluggish, chronic infections if properly combined with rest, nutrition, fresh air and other therapeutic measures to improve general systemic conditions, and in many such cases has justified its use if, of course, the vaccines in these latter cases were autogenous. In subacute conditions, such as endocarditis in which the focus cannot be surgically reached, and in which bacteria appear in the blood, autogenous vaccination has some theoretical justification, but has, in our own experience, and in an unprejudiced study of the literature, not impressed us as having given much hopeful therapeutic result. In acute infectious diseases any effect that has been claimed for vaccines, we believe, has been due to the nonspecific reactions which are discussed in a later paragraph and in no sense to a specific immunizing effect of the injected bacteria.

In prophylactic vaccination, such as typhoid, plague, cholera, etc., described in the subsequent sections, homologous vaccines made from stock cultures are, of course, necessary, and have justified themselves by brilliant results. In the treatment of existing infections, whatever their nature, the use of stock commercial cultures, or stock cultures kept in laboratories have very little specific value, largely because of the many antigenic varieties existing within the same species of most of the bacteria that give rise to infections in which this question usually arises. Autogenous vaccines, only, can be expected to give any sort of result. The random use of stock vaccines without laboratory diagnosis and without control is, of course, an entirely unjustifiable procedure in cases of this kind.

Moreover, the control of vaccine treatment by opsonic index determinations, as suggested by Wright and his coworkers, is not a

practicable method for clinical application. The measurements, as we have pointed out, are entirely too laborious and, therefore, expensive. Their accuracy is subject to so many uncontrollable variables that there is nothing to be gained for the patient, and equally nothing to be gained for the physician in his judgment of the case. Accurate and skilled clinical supervision is of far greater value.

As we have said before, the opinions expressed above are given with the purpose of stating as clearly as we can the logic of vaccine therapy as we see it at present. The next ten years of clinical experience may largely modify these views. One thing is certain, however, and that is that the problem can only be settled if treatment by this method is undertaken with the guidance of an accurate bacteriological diagnosis, and with bacteriological control of the individual case, so that, when occasion arises, estimations of antibodies can be made.

THE PRODUCTION AND STANDARDIZATION OF VACCINES

Vaccines in the sense of Wright consist merely of killed cultures of the bacteria with which the patient is infected. In all cases it is extremely desirable to make such vaccines "autogenous," by which we mean that the organism used is one which has been isolated from the case. The difference between various strains of the same species of bacteria seems to make this imperative whenever it is at all possible. The recent investigations of Neufeld and Haendel in determining that there are a number of types of pneumococcus which are antigenically distinct illustrates this point. The same principle is made clear by the recent work of Rosenau on the streptococcus-pneumococcus group. Especially important is Rosenau's observation that a pneumococcus which he had been able to transform culturally by special methods was found to be altered also in its reaction to agglutinins.

In the development of prophylactic methods of vaccination against epidemic disease like typhoid, cholera, plague, etc., many different methods of antigen preparation have been developed. In typhoid prophylaxis the bacteria have been used dead, living, and sensitized, and even extracts have been employed. In cholera the early use of living cultures by Ferran has given way, in the hands of Kolle and others, to that of dead bacterial emulsions. In plague and a number of other conditions the impression seems to be general that the bacteria should be used in the living, but attenuated, state. Special methods which have been developed in these cases are discussed in another section.

In treatment of developed diseases with vaccines the method most commonly used is that which has been introduced by Wright, namely, the use of dead cultures. In his earlier experiments Wright culti-

vated the bacteria on agar slants for about 24 hours, then washed off the growth with 10 c. c. of sterile salt solution. It will be well to describe in detail the preparation of such a vaccine.

The bacteria must be isolated from the patient by the usual method of plate cultivation and colony fishing on suitable media. We do not think that any satisfactory substitute for careful isolation by plating has been devised. After a pure culture of the organism has been obtained this is grown on relatively large surfaces of agar, glucose-agar, or ascitic agar, as the case may require. These cultivations may be made in Kolle flasks or, as Wright and others have suggested, on large agar surfaces obtained when the culture medium is allowed to harden in a square 3-oz. medicine bottle laid on its side. Any device of this kind in which a large surface of agar is exposed may be used.

After suitable growth of the micro-organisms has taken place, 24-48 hours, the growth is gently washed off with 10 c. c. or more of sterilized salt solution. Care must be taken to do this in such a way that no agar is drawn away with the emulsion. The thick emulsion so obtained is removed from the culture bottle with sterile nipple pipettes or Pasteur pipettes and transferred to a sterile thick-walled test tube into which glass beads have been placed. By drawing out the neck of this test tube in the flame a glass capsule is formed, in which we now have our so-called stock emulsion. (See figure.)

Capsule Made of Test Tube to Hold Stock Vaccine Emulsion from Which Dilutions are Made.

(It is well to keep capsule open to capillary tip until it has cooled off, otherwise it may crack when quickly cooled.)

The next thing to be done is to standardize this stock emulsion, or, in other words, determine approximately the number of bacteria to the cubic centimeter. There are a number of methods by which this can be done.

The method most extensively used by Wright and his followers was that in which the bacteria are counted against red blood cells. The bacteria in the capsule are shaken thoroughly with glass beads so that clumps may be broken up and even distribution obtained. A little of the emulsion is then put into a clean watch glass, a step which can be accomplished most easily by breaking off the tip of the drawn-out part of the capsule, tilting it very gently and heating the closed end over a small flame, so that some of the emulsion will be driven out by the expanding air. With a nipple pipette marked about an inch from the tip, as in the taking of an opsonic index, a little of the emulsion is drawn up. This is placed into another clean watch glass and is mixed with about 2 volumes of salt solution and

one volume of blood from the finger, these quantities being measured with the same nipple pipette. We then have a mixture in which, in a total of 4 volumes, there are equal parts of blood and of bacterial emulsion. After this emulsion has been thoroughly mixed by drawing in and out through the nipple pipette smears are made on slides and stained with Jenner or any other suitable blood and bacterial stain. Under the field of the microscope the ratio between the bacteria and blood cells is then determined, and from our knowledge of the number of the red blood cells in this blood to each c. mm. we can easily calculate the number of bacteria to the c. mm. or c. c.[2]

A more accurate method of enumerating the bacteria in a suspension to be used for vaccine is by direct count of an accurately made dilution in a hemocytometer chamber, as was first suggested by Malory and Wright in 1908.[3] The bacterial suspension is diluted in blood-counting pipettes, 1-20 to 1-100 dilutions of thick bacterial suspensions being as a rule satisfactory. As a diluent one may use either salt solution or some dilute anilin dye, such as one made by mixing one part alcoholic methylene blue with 40 parts of 1 per cent. carbolic acid. The dilute suspension is then placed in an ordinary Thoma-Zeiss chamber, which was designed for counting blood platelets and has a depth of 0.02 mm. This enables one to use an oil immersion lens or high power dry system with a short working distance. From such a count one may readily estimate the number of bacteria in the original suspension; for example, if 20 squares in the Helber-Zeiss chamber are counted the result gives the number of bacteria in 0.001 c. mm.[4]

Microscopic Field as Seen in Standardization of Vaccines by Wright's Method.

Another method of standardization of vaccines which is suffi-

[2] For such counts it is convenient to contract the field of the microscope by using a diaphragm or simply marking a circle on the eyepiece with a grease pencil.

[3] Malory and Wright. "Pathological Technique," 4th Ed., New York, 1908.

[4] Glynn, Powell, Rees, and Cox. *Jour. Path. and Bact.*, Vol. 18, 1914, p. 379.

ciently accurate for clinical purposes is that of Hopkins, which consists in measuring the volume of the sediment [5] after centrifugalizing the preparation under standard conditions in a graduated tube. The tubes may be made with a capacity of 10 to 15 c. c. with a capillary tip about one inch in length, having a capacity of about 0.05 c. c. graduated in 0.01 c. c. The bacterial suspension, after being filtered through sterile cotton to remove fragments of the agar or other foreign bodies, is centrifugalized in such a tube for half an hour at about 2,800 revolutions a minute. The supernatant fluid and bacteria are removed down to the 0.5 c. c. mark and the sediment resuspended in 5 c. c. sterile salt solution by means of a capillary pipette which gives a 1 per cent. suspension. 0.05 c. c. of streptococci sedimented in this way represent quite constantly 16 mm. of dried bacterial substance. The number of organisms per cubic centimeter contained in 1 per cent. suspension in this way are as follows:

Streptoccocus aureus and albus	10 billion
Streptoccocus	8 "
Gonococcus	8 "
Pneumococcus (capsulated)	2.5 "
Bacillus typhosus	8 "
Bacillus coli	4 "

After the vaccines have been standardized suitable dilutions can be made in salt solution to which 0.5 per cent. carbolic acid or some other antiseptic has been added. The dilutions are usually so made that from 100 to 500 million bacteria are contained in the cubic centimeter, this being a suitable initial dose of most organisms. The dilutions are placed in sterile bottles containing beads and fitted with rubber caps. These bottles can be shaken before use, the emulsion thoroughly distributed, and the desired quantity can be taken out with a sterile hypodermic syringe thrust through the rubber cap after this has been covered with a small amount of lysol or strong carbolic (see figure). After the dilutions have been made both these and the stock vaccines should be sterilized. Some workers sterilize always the stock vaccines and make the dilutions with asceptic proportions in such a way that no further sterilization is necessary. This is preferable because the less heat that is applied the better it is for the preservation of their antigenic properties—sterilization is usually accomplished by heat in the water bath. Wassermann's earlier technique called for heating to 60° C. for one hour for a number of consecutive days. It is generally considered at the

VACCINE STOCK EMULSION IN RUBBER TOP BOTTLE.

[5] Hopkins. *Jour. A. M. A.*, Vol. 60, 1913, p. 1615.

present time that it is better not to heat above 55° C. After the vaccine has been heated its sterility must be controlled to aerobic and anaerobic cultivation, and possibly by animal inoculation, although, except in special cases, this is unnecessary. Some workers, especially when the vaccine is to be extensively used, as in typhoid immunization, inject some of the vaccine into white mice to exclude the possibility of contamination with tetanus. In such cases also it is not inadvisable to test out the antigenic value of the vaccine upon animals, measuring the agglutinins, etc., which result from a number of inoculations. In the preparation of a therapeutic vaccine where speed is required this of course is not feasible. Moreover, it is unnecessary in view of the fact that we wish to inject that particular organism into the patient from whom it has been cultivated. Whatever its antigenic value may be from animal experiments, it is preferable for the given purpose to any other strain.

Sensitized vaccines are easily made by exposing emulsions of the bacteria to moderate amounts of a strong immune serum which has been heated to 56° C. to destroy the complement. Bacteria will usually agglutinate under these circumstances and can easily be centrifugalized to the bottom. The excess serum is then washed off and the bacteria emulsified as in the case of the preparation of vaccines with dead organisms.

Prophylactic Immunization in Typhoid and Paratyphoid Fever.—The first attempt to inoculate human beings with typhoid bacilli with the intention of producing prophylactic active immunization was probably that made by Pfeiffer and Kolle [6] in 1896. During the same year also Wright [7] made similar studies in England, and soon after this he reported upon the development of antibodies in the blood of 17 people inoculated with typhoid. By these studies it was shown that human beings could be inoculated with dead typhoid bacilli without danger, and this logically led to the attempt to vaccinate human beings on a large scale.

It is hardly necessary to dwell upon the desirability of such a procedure. From tables recently published by Russell [8] we take the information that, in our own Spanish American war, 20,738 cases of typhoid with 1,580 deaths occurred in a total enlistment of 107,-973. In this entire war 243 men were killed in action or died of their wounds, while almost 7 times as many died of typhoid fever. In the British army during the Boer war there were over 75,000 cases of typhoid in 380,000 men, and in the Russian army during the Russo-Japanese war over 17,000 cases of typhoid occurred, over half as many as the number of men killed in action. Such appalling figures leave no possible doubt as to the desirability of prophylactic im-

[6] Pfeiffer and Kolle. *Deutsche med. Woch.*, Vol. 22, 1896, p. 735.
[7] Wright. *Brit. Med. Jour.*, January, 1897, p. 256.
[8] Russell. *Amer. Jour. of Med. Sc.*, Vol. 146, December, 1913.

munization in armies, and there can be little question that typhoid fever is sufficiently prevalent in many parts of the civilized world to encourage prophylactic immunization of individuals, even when not living under the especially dangerous conditions of camps.

Following the preliminary studies of Pfeiffer and Kolle and of Wright extensive practical studies of vaccination were made in the German colonial army during the Herrero war, and by British bacteriologists during the Boer war. Leishmann [9] also studied carefully the results of vaccination among regiments of the British army in India.

The vaccine employed by Wright and his associates in England consisted of broth cultures of a typhoid bacillus killed by exposure to 53° C., and by the further addition of 0.4 per cent. of lysol. The German vaccine consisted of emulsified agar cultures similarly killed.

The results obtained with these vaccines, although encouraging, were not as striking as had been hoped. Russell [10] summarizes the earlier attempts by stating that the morbidity was reduced to about one half among vaccinated persons with a slightly greater reduction of mortality. The last-named writer also attributes the early failures to the overheating of the vaccines with a consequent reduction of their antigenic properties, and to timidity in their administration resulting from Wright's fear of a negative phase. Russell, of the United States Army Medical Corps, made a most extensive study of typhoid vaccination in this country. After careful consideration of the methods of others he produces his vaccines as follows: A single strain of typhoid bacilli was used (culture Rawlings obtained from England), and this grown on agar in Kolle flasks for 18 hours. The purity of the culture was tested out both morphologically and by transplantation upon the double sugar media devised by Russell. Agglutination tests are also made. After 18 hours the growth was washed off in small quantities of salt solution and the emulsion heated in a water bath for one hour at 53° C.; it was then diluted with sterile salt solution to a concentration of one billion bacteria to a cubic centimeter. Then 0.25 per cent. of tricresol was added. Before use the safety of the vaccine was ascertained both by aerobic and anaerobic cultivation and by the injection into mice and guinea pigs of considerable quantities for the exclusion of possible tetanus contamination. The efficiency of the vaccine was then tested by injecting rabbits with three doses at 10 day intervals, and determining the resulting agglutinating power.

With these vaccines under the direction of the United States Army Medical Corps the troops mobilized in Texas, California, and along the Mexican border were treated. Compulsory vaccination was

[9] Leishmann. *Glasgow Med. Jour.*, Vol. 77, 1912, p. 408, cited from Russell.

[10] Russell, *Amer. Jour. Med. Sc.*, Vol. 146, December, 1913.

established in March, 1911, and the results as reported by Russell have fully justified the measure. The following table taken from Russell's paper will illustrate the results obtained:

Typhoid Fever. Officers and Enlisted Men, United States Army

	Yr.	Jan.	Feb.	Mar.	Apr.	May	June	July	Aug.	Sept.	Oct.	Nov.	Dec.	Totals for 9 months
Voluntary	1908	5	6	4	2	3	11	14	31	25	26	12	8	101
	1909	4	10	6	4	11	15	26	14	16	45	20	6	106
Compulsory	1910	8	11	1	4	2	6	12	27	21	16	20	11	92
	1911	3	3	3	7	4	4	4	7	4	4	1	0	39
	1912	1	2	2	0	0	3	1	3	1	4	0	1	13
	1913	0	0	0	0	0	0	0	0	0	0	0	0	0

Paratyphoid fever included in figures for 1908, but excluded in other years. Cases paratyphoid 1909, 3; 1910, 3; 1911, 2; 1912, 3; 1913, 0.

We have mentioned in another place that Metchnikoff and Besredka in their studies on typhoid vaccination in the chimpanzee have concluded that very little protective value resided in vaccination with dead typhoid vaccines, whereas animals vaccinated with small amounts of living cultures were very efficiently protected. Metchnikoff and Besredka adopted finally the method of immunizing with living sensitized vaccines. By this is meant typhoid bacilli that have been exposed to the action of heated immune serum, or, in other words, typhoid bacilli that have absorbed specific antibodies. There is no question as to the efficiency of this form of vaccination. The method of employing sensitized bacteria for these purposes utilized by Besredka in the case of plague has unquestionably won an important place in active immunization. However, the results of Russell and others seems to indicate that in human beings the use of dead vaccines is certainly of considerable value, and there are certain practical objections to the use of living vaccines in immunization of large numbers of people as in armies to which Russell calls attention. In the first place, living vaccines cannot be stored for any considerable period, and may become a source of possible infection by mouth if carelessly handled; furthermore, contamination is not so easily ruled out in the case of living vaccines when used on a large scale, and it is not possible at present to require compulsory vaccination with living bacteria. The choice of strains is important. For vaccines in the British and American armies the Rawlings strain originally used by Wright is employed at present. The work of Weiss, Hooker and others recently has shown that perhaps we may have to use for best results a polyvalent typhoid injection, and that in immunizing with paratyphoid B, a polyvalent vaccine will be necessary

eventually. The paratyphoid A strains are so uniform antigenically that this will be unnecessary in their case.

Gay has recommended the use of sensitized killed vaccines. He controls the efficiency of his vaccines by testing them out on rabbits in which typhoid septicemia has been produced by inoculation with cultures grown on rabbit blood agar. These vaccines have not yet been used upon sufficient numbers to justify conclusions. It would seem, however, that any one of the methods mentioned must possess considerable value, since they all represent merely slight variations of the same procedure. The method at present used in the German, British, and American armies, namely, vaccination with dead cultures, seems certainly, according to Russell's statistical studies, to have yielded excellent results and recommends itself by its extreme simplicity and safety.

The proof of this was brought conclusively during the last war by the freedom of armies from typhoid fever, in spite of the most unfavorable and unpreventable sanitary difficulties existing during prolonged battles in areas of concentration.

Since, therefore, the methods first recommended by Russell and others have stood the actual test on many millions of men, it seems unadvisable to experiment on a large scale for the present with other methods.

The vaccine as prepared at the present time, is made up of typhoid, paratyphoid A and B, so distributed that each cubic centimeter contains one million typhoid bacilli and 750 million of each of the two paratyphoids. Three doses are given at 7 to 10 day intervals, the first one of 1/2 c. c. of this vaccine, and the second and third of 1 c. c. each. In cases of children or adults in which care is indicated, the dosage can be reduced and then the entire amount distributed in a larger number of injections. Indeed, we have, on a number of occasions, given the vaccine in 4 and 5 injections, 6 to 7 days apart, in amounts ranging from 0.25 to 0.75 c. c., in the hope of getting more thorough immunity in cases in which there was time for this procedure.

In vaccinating it is extremely important not to put the vaccine into a small vein and not to inject it intramuscularly. It should be administered subcutaneously so that the finger feels a slight bulge under the needle. Intracutaneous injection should also be avoided.

There is always a local reaction which may attain an area of 5 or 6 c. c. in diameter over which there is redness, swelling and some pain. There may be swelling of regional lymph nodes. Suppuration should not occur in vaccination properly performed. General reactions vary in different individuals. In most healthy adults the first injection gives little trouble. But after the second or third, there may be headache, nausea and temperature, coming on several hours after the injection. In some cases these symptoms may persist

for several days, and the patient feel very miserable, but no severe harm is done. If, as occasionally may happen, the injection passes into a vein, there may be very violent symptoms within 20 minutes after injection, consisting of a chill, followed by temperature, vomiting and nausea, and a condition almost bordering on collapse. We have seen two such cases in both of which the symptoms were alarming. Great attention should, therefore, be paid to this point.

Typhoid vaccine is contraindicated in chronic nephritis, in any acute illness in which there is fever, and during convalescence. There was at one time very much literature on the dangers of typhoid vaccination in cases of tuberculosis. The judgment of Russell, Nichols [11] and others who have had most experience with vaccination, is that tuberculosis is not a contraindication if there is sufficient reason for vaccination, that is, danger of infection. They state that neither typhoid fever nor vaccination have any harmful effects on the progress of tuberculosis. However, should a tuberculous case be presented for vaccination, it is well to withhold vaccination if there are acute symptoms and temperature, and to wait until these have subsided.

Therapeutic Vaccine Treatment in Typhoid Fever.—A number of workers have attempted to treat typhoid fever after it occurred with typhoid vaccine sensitized and unsensitized. Frankel did this as early as 1893. He was followed by Petruschky [12] and Netter and in 1912 Ichikawa began the intravenous injection of typhoid vaccine in patients with the rather astonishing results of obtaining in many cases rapid falling of temperature and often apparent shortening of the disease. Boinet in 1912 obtained similar results and recently the same thing has been done by Gay.[13]

Of these writers the only one who recognized immediately that perhaps the intravenous injection of such vaccines was not due to purely specific activity was Ichikawa, who thought the paratyphoid bacilli injected into typhoid cases often produced similar results, and soon after this Kraus obtained similar degrees of temperature and favorable results by injecting colon bacilli into typhoid patients, into a few cases of pyocyaneus infection, and into streptococcus septicemias.

With Jobling and others we have believed from the beginning that at least an important factor in the activity of these vaccines was non-specific, due perhaps to a rapid mobilization of leukocytes and of ferments. This is discussed in another section.

At any rate the therapeutic results obtained justify the cautious continuance of such intensive treatment in various diseases.

Active Prophylactic Immunization in Cholera.—Attempts to

[11] Russell and Nichols. *Jour. A. M. A.*, Vol. 76, 1921, p. 177.
[12] Petruschky. *Centralbl. f. Bakt.*, 1, Vol. 19, 1896.
[13] Gay. *Arch. of Int. Med.*, 1914.

protect human beings against cholera by prophylactic vaccination were made as early as 1885 by Ferran,[14] a pupil of Pasteur. At the time at which Ferran's experiments were done little was known regarding the production of immunity with killed cultures or with bacterial extracts, and Ferran, under the influence of the French school and its endeavors to immunize with living attenuated organisms, applied similar methods to cholera. First experimenting with guinea pigs, he soon applied his method to human beings, inoculating them with small quantities of living broth cultures of cholera spirilla. In many of his experiments he gave, at the first injection, 8 drops of a fresh broth culture, following this after 8 days with 0.5 c. c. of a similar culture. There is no reason why Ferran's method should not have yielded excellent results. However, it is stated that he worked with impure cultures, and other observers, notably Nikati and Rietsch, van Ermengen, da Lara, and others, failed to obtain encouragement in their subsequent investigation of this method of vaccination.

The method which Haffkine [15] worked out some years after Ferran's experiments also depended upon the injection of living cultures, but Haffkine attempted, by a rather elaborate technique, to produce two separate vaccines, one attenuated, the other enhanced in virulence. Attenuation was accomplished by growing the cholera spirilla at a temperature of 39° C. in broth over the surface of which a constant stream of sterile air was passed. Under these conditions the first crop of cholera organisms died rapidly, but Haffkine practiced reinoculation into new broth flasks before complete death of the original culture had taken place; after a series of generations of cultivation in this way he obtained cultures which produced merely temporary and slight edema when injected under the skin of guinea pigs. This weakened virus was used for the first inoculation.

He enhanced the virulence of cholera cultures with the purpose of producing a strain of maximum potency comparable to virus fixe. His procedure was as follows:

a. Giving an animal an intraperitoneal injection of cholera spirilla larger than the fatal dose.

b. Taking out the peritoneal exudate and exposing it for a few hours to the air.

c. Injecting this exudate into another animal and treating the resulting peritoneal exudate in the same way.

After a series of such animal passages he claims to have obtained a virus of great virulence, and this is his second and stronger vaccine.

In applying the method to human beings Haffkine planted the cholera spirilla upon agar slants of the standard size, emulsified the growths in sterile water, and injected 1/5 to 1/20 c. c. of such a

[14] Ferran. *Compt. Rendu de l'Acad. des Sc.*, 1885.

[15] Haffkine. *Lancet*, February, 1893; *Brit. Med. Jour.*, December, 1895.

culture, using first the weak vaccine and five days later a more virulent culture.

Beginning his work as early as 1893, Haffkine and others vaccinated as many as 40,000 people in India. On the whole, the results obtained were very encouraging. It is a question, however, whether or not his method is unnecessarily complicated. In the light of our more recent knowledge concerning cholera immunity it seems likely that the importance which Haffkine attached to the virulence of the cholera culture used for injection was exaggerated, and we have reason to believe that simple immunization with killed cultures may produce results fully as efficacious. After all, we could not expect, at least at present, to produce by active artificial immunization an immunity as permanent as that which results from an attack of the disease. Concerning the reasons for the acquisition of such permanent immunity we have as yet little knowledge. Even Haffkine's method of inoculation with living virus does not, by his own estimation, last longer than possibly two years. It is therefore likely that prophylactic immunization in cholera is efficacious by reason of the appearance in the blood serum of the specific bactericidal and opsonic substances by which the small numbers of cholera organisms entering during spontaneous infection can be disposed of before a foothold in the body is gained.

Tamancheff later used Haffkine's method, but killed the cultures by the addition of a 0.5 per cent. solution of carbolic acid.

Kolle [16] later recommended the injection of dead cholera organisms, maintaining that a single injection of about 2 milligrams of a culture killed by exposure to 50° C. for a few minutes, and by the addition of 0.5 per cent. of phenol, is sufficient to immunize successfully. Good results with Kolle's method have been reported from Japan.

Strong,[17] also proceeding from the idea that the immunizing antigen is present, as such, within the cell body of the cholera spirilla, recommends the injection of autolytic products obtained by digesting cholera spirilla in aqueous suspension and filtering. He prepared his "prophylactic" by growing the organisms upon agar, then suspending the growth in sterile water and keeping it at 60° C. for from one to twenty-four hours. The mixture was then exposed to 37° C. for from two to five days and filtered through Reichel filters. One to 5 c. c. of this was used in his experiments upon human beings.

According to Teague [18] and others, none of the more complicated modifications of cholera vaccine have any particular advantage over simple salt solution suspensions of young agar cultures killed by heat-

[16] Kolle. *Deutsche med. Woch.*, 1897, No. 1.
[17] Strong. *Jour. Inf. Dis.*, Vol. 2, 1905.
[18] Teague. *Jour. A. M. A.*, Vol. 76, 1921, p. 243.

ing one hour at 53° C. The vaccine made in the Government Laboratories in Bombay contains, according to Teague, eight thousand million organisms per cubic centimeter, and 0.5 c. c. administered subcutaneously at the first dose, 1 c. c. at the second. It has been used extensively in India, and reports from there seem to indicate favorable results. During the last war, Teague states that several million men in the Austrian and German armies were vaccinated, and many thousands of them spent months in districts where cholera was present. A relatively small amount of cholera occurred.

Contraindications in cholera are similar to those stated for typhoid fever. Vaccine treatment in acute attacks of cholera is useless, as far as we know.

Prophylactic Immunization Against Plague.—The first attempts to immunize human beings prophylactically against plague were those of Haffkine.[19] The first vaccinations were carried out with broth cultures killed at 65° C. He tested out his vaccines on a large scale in Bombay, and obtained apparently promising results. In a plague epidemic occurring in a Bombay prison only 2 of 151 vaccinated persons became ill, and neither of these died; whereas, of 177 unvaccinated persons 12 became ill and 6 died. In large series of vaccinated people only 1.8 per cent. were infected with plague, with a mortality of 0.4 per cent. for the total, whereas of unvaccinated individuals in the same epidemic 7.7 per cent. fell victim to the disease, with a mortality of 4.7 per cent.

The German Plague Commission, consisting of Gaffky, Pfeiffer, and Dieudonné, recommend a vaccine of killed agar cultures. Kolle and Otto,[20] basing their earlier results upon experiments carried out with monkeys, mice, guinea pigs, and rats, have come to the conclusion that vaccination with dead plague cultures is much inferior to that obtained when attenuated living cultures are used. The same conclusion has been reached by Kolle and Strong.[21] Kolle and Otto found that the immunization of animals with large doses of killed agar cultures of plague bacilli and with Haffkine's prophylactic did not protect them against subsequent inoculation with virulent cultures.

Strong [22] subsequently made a very careful comparative study of the various methods of plague vaccination, and concluded that the most efficient method is immunization with attenuated living cultures. He showed that when carefully done this method can be safely employed in human beings, but admits that his work must be as yet considered as experimental and further studied before it can be universally employed.

[19] Haffkine. *Bull. de l'Inst. Past.*, Vol. 4, 1906, No. 20, p. 825.

[20] Kolle and Otto. *Deutsche med. Woch.*, 1903, p. 493, and *Zeitschr. f. Hyg.*, Vol. 45, 1903.

[21] Kolle and Strong. *Deutsche med. Woch.*, Vol. 32, 1906, p. 413.

[22] Strong. *Jour. Med. Res.*, N. S., 13, 1908.

Besredka [23] has advised the use of sensitized dead plague cultures, claiming, from animal experimentation, that such vaccines produce an efficient and relatively durable immunity.

Rowland [24] confirms the immunizing properties of Besredka's vaccines in plague, and believes that the antigenic properties of the plague bacillus are attached to the bacterial nucleoproteins, and can be extracted with these. Rowland prepares a vaccine by the treatment of the moist bacteria with enough anhydrous sodium sulphate to combine with all the water present, freezing and thawing the mixtures, then filtering off the bacterial deposits at 37° C., and extracting them with water. The solution so obtained was fatal to rats in small quantities and afforded substantial protection, reducing the mortality on subsequent inoculation of a standard culture from 80 to 10 per cent.

Haffkine's virus which consists of broth cultures grown for six weeks at room temperature and then killed by heating to 65° C., for one half hour and the addition of 0.5 per cent. carbolic acid, is still extensively used in India and other British possessions, but is gradually being replaced by simple saline suspensions of 24 hour agar cultures heated for one hour at 65° C. The latter type of vaccine is stated by Teague as being more effective in producing immunity in laboratory animals.

The efficiency of the vaccine can best be judged by reports from the British Indian Plague Commission. In the report of 1911, prepared by Major Glen Liston, it is stated that 1,211,170 doses of vaccine were distributed. His figures of comparison between the inoculated and uninoculated, as far as they could be gathered reliably, are very favorable to the efficiency of the vaccine. A comparative table indicates the degree to which this is true.

Comparative Morbidity and Mortality from Plague Among Inoculated and Uninoculated [25]

Inoculated		Not Inoculated	
Population	118,148	Population	321,621
Attacks	941	Attacks	11,041
Deaths	371	Deaths	8,695
Incidence, per 1,000.....	7.96	Incidence, per 1,000.....	34.4
Case mortality, per cent.	39.5	Case mortality, per cent.	78.6

Since a population like that of India offers so many difficulties to statistical study, some of the most important contributions to our knowledge of plague vaccination are obtained from small outbreaks in towns where the population has been under the observation of

[23] Besredka. *Bull. de l'Inst. Past.*, Vol. 8, 1910.
[24] Rowland. *Jour. Hyg.*, Vol. 12, 1912, p. 344.
[25] Teague. Cited from *Jour. A. M. A.*, Vol. 76, 1921, p. 244.

individual physicians for very long periods. Thus, Beals [26] inoculated in 1916-1917 4,378 people in the town of Wai, Bombay. In that year, of the 6,000 uninoculated people in Wai, 270 got the disease, and 167 died. Among 4,378 inoculated persons, there were only 39 attacks and 10 deaths. In 1917-1918, among the uninoculated, less than 6,000 people, there were 76 attacks of plague and 47 deaths, while with the 4,081 inoculated there were 17 attacks and only two deaths.

Prophylactic Vaccination in Pneumonia.—Owing to the high death rate from pneumonia in mining camps, such as those in South Africa, in Panama, and among soldiers during the war, it has seemed desirable to work out a method of vaccination against pneumonia on a large scale. The most extensive experiments on record in this direction are those of Cecil and Austin in this country, and the preceding ones of Lister in South Africa. Lister prepared a vaccine by using his own types A, B and C (B and C corresponding, respectively, to II and I of our classification), and injected these in salt solution suspensions in amounts of from six to seven billion at a dose. His first injections were done intravenously. Later, he used subcutaneous inoculations, giving only three doses of two billion each at weekly intervals. His results were inconclusive but encouraging. Cecil and Austin,[27] and Cecil and Vaughan [28] have continued this work in America.

At Camp Upton during the war, they vaccinated over 12,000 men against Types I, II and III, administering three or four doses of a saline vaccine at intervals of 5 to 7 days, with a total dosage of six to nine billion of Types I and II, and four to six billion of Type III. Forty per cent. of the Camp were vaccinated. Among the unvaccinated, there were 173 cases of pneumonia of all types, and among the 40 per cent. vaccinated, there were 17 cases. A similar experiment was carried out at Camp Wheeler, the only difference being that a lipo-vaccine, that is, a suspension of pneumococci in neutral fat, was used. Over 13,000, or 80 per cent., of the men were vaccinated. Here the period of post-vaccination observation was longer, that is, two to three months. During this period there were 32 cases, among the 80 per cent. vaccinated, and 42 among one-fifth of the men who were unvaccinated. These figures, while encouraging, are not as convincing as they should be in order that we can pass definitely favorable judgment.

Of value to such judgment, however, are the monkey experiments of Cecil and Blake.[29] They found that the ordinary small subcutaneous doses of vaccine are not sufficient to protect monkeys

[26] Beals. *Jour. A. M. A.*, Vol. 75, 1920, p. 955.
[27] Cecil and Austin. *Jour. Exp. Med.*, Vol. 28, 1918, p. 19.
[28] Cecil and Vaughan. *Jour. Exp. Med.*, Vol. 29, 1919, p. 457.
[29] Cecil and Blake. *Jour. Exp. Med.*, Vol. 31, 1920, p. 519.

against a subsequent attack of pneumonia, but that they would modify the course of the disease favorably. Subsequently, Cecil and Stephen [30] found that if large subcutaneous or intravenous doses of pneumococcus vaccine were used, prophylactic immunization of the monkeys were successful.

These workers believe that there is a large field for pneumonia vaccination under circumstances where epidemics are expected, such as the conditions of concentration camps during cold weather mentioned above, and perhaps in the course of the high pneumonia mortality encountered in the second and tertiary waves of influenza epidemics.

The vaccine is contraindicated in acute infections, in pulmonary tuberculosis and nephritis. Also, it should not be given to patients with chronic heart disease, or pregnant women after the fifth month.

The active treatment of pneumonia as it has appeared with vaccines, is, so far, without experimental or therapeutic foundation.

Prophylactic Vaccination in Influenza.—Since we are not sure about the relationship of the Pfeiffer bacillus to epidemic influenza, prophylactic immunization with this organism is not entirely logical. Nevertheless, since the most severe complications in secondary waves of such epidemics are unquestionably due to influenza bacilli, streptococci and pneumococci, many attempts have been made to at least protect against serious complications by prophylactic vaccination. Extensive experiments were made on this during the last great epidemic. Eyre and Lowe [31] inoculated 16,000 men, leaving 5,700 controls which were either uninoculated or received only one dose. These experiments were done with a mixed vaccine containing pneumococci, streptococci, influenza bacilli, staphylococcus aureus, micrococcus catarrhalis, Friedlander bacillus, and a bacillus which they called B. septus. They obtained a slightly greater incidence among the uninoculated, that is, 4.1 per cent., as against an incidence of 1.3 among the inoculated. Cadham [32] in 1919 reported upon the use of mixed vaccines of streptococcus, pneumococcus and influenza bacillus upon soldiers. He claimed that the incidence of pneumonia was one-half and the mortality less than one-third among the inoculated as compared with the uninoculated. Wirgman [33] also in 1919, reported observations on 11,000 people of whom 800 were inoculated in November and December, 1918, and January, 1919. He, too, reported favorable results in that the incidence among the inoculated was one-half of that occurring among the uninoculated, and that there was no death rate among the inoculated as against those who had not been treated. In spite of these ap-

[30] Cecil and Stephen. *Jour. A. M. A.*, Vol. 76, 1921, p. 178.
[31] Eyre and Lowe. *Lancet*, Oct. 12, 1918.
[32] Cadham. *Lancet*, Vol. 1, 1919, p. 885.
[33] Wirgman. *Lancet*, Vol. 1, 1919, p. 357, and *Lancet*, Vol. 2, 1918, p. 324.

parently favorable results, a critical survey of the situation by McCoy [34] points out many significant sources of error. The chief one of these is the fact that the inoculations had usually been made during the progress of an epidemic, while the cases among the general population or control groups, are calculated from the beginnings of the epidemic. Also, the vaccine cannot be expected to have any appreciable prophylactic effects in less than 7 to 10 days. He has been able to collect, furthermore, a considerable number of observations such as the one done on the Naval personnel at the Pelham Bay Training Station (*U. S. Naval Bulletin* No. 50 and No. 51), and the experiments of Hinton and Kane [35] in an epileptic colony in which there were absolutely no appreciable results. McCoy's opinion is that in spite of a generally favorable impression, the evidence so far, whenever experiments have been properly controlled in every particular, does not demonstrate any effects on either incidence or mortality.

The Use of Vaccines in Streptococcus Infections.—In streptococcus infections the problem of vaccine treatment is very similar to that encountered in staphylococcus lesions. It is less frequently applicable, largely because the conditions caused by the streptococcus are usually much more acute and severe, and under such circumstances vaccine treatment has very little purpose. When, however, streptococcus infections are sluggish, connected with old sinuses or sequestra, wounds that will not heal, or in the form of furuncles, vaccine treatment may be applied with some hope of raising the general resistance of the patient.

Here, again, it is necessary to use a strain obtained from the patient, since not only the streptococcus viridans, but the hemolytic streptococci, as well, are antigenically heterologous. In the case of the viridans, it is rare to find two entirely homologous strains. They vary in many subgroups like the Type IV pneumococci. The recent work of Dochez, Avery and Lancefield,[36] moreover, has shown that the hemolytic streptococci can be divided into groups. Stock or commercial vaccines are, therefore, useless, except for a certain amount of non-specific effect, such as that described in another section. The vaccines are made in the usual manner, sterilized, tested, and standardized to about twenty million per cubic centimeter.

Vaccination in Infections with the Staphylococcus Pyogenes Aureus.—In no infection has vaccination been so extensively attempted as in the various staphylococcus lesions of the skin and subcutaneous tissues commonly spoken of as boils and furuncles. This subject has been discussed to a considerable extent in the chapter

[34] McCoy. *Jour. A. M. A.*, Vol. 73, 1919, p. 401.

[35] Hinton and Kane. *Bimonthly Bulletin*, State Bd. Health, Mass., 28, 1919, p. 6.

[36] Dochez, Avery and Lancefield. *Jour. Exp. Med.*, Vol. 30, 1919, p. 179.

on opsonic index in which Wright's experiments on therapeutic vaccination in these conditions have been detailed. The immunological circumstances in these infections are peculiar in that a considerable degree of local immunity is probably developed by the tissues immediately surrounding the staphylococcus focus; for, as is well known, a patient suffering from boils may be completely getting the better of an infection at one site, while a few inches away another focus is starting and progressing vigorously. There are a number of possible explanations for this, in addition to local resistance. It may be that the leukocytic concentration at the focus, due to purely chemotactic influences, accumulates at the point of attack a defensive mechanism which is not dependent upon acquired powers of resistance of the fixed tissues at this point; from this it follows that if we could hasten the accumulation of leukocytes at the focus, by artificial means, the lesion might be arrested without any fundamental increase of immunity. At any rate, the fact remains that boils may follow each other, one upon the heels of the other, or may come out in small simultaneous crops with intervals of a week or more between their appearance. Since staphylococci of pathogenic varieties are probably in constant contact with the skin of human beings, getting into small abrasions, hair folicles, etc., it is likely that the incidence of boils is not merely due to accidental contact or uncleanliness in a person of normal resistance. It is more than likely that, in addition to such contact, a lowered resistance against staphylococci must exist, at least in the usual case. It is noticeable that boils occur particularly in individuals suffering from some systemic disease such as nephritis or diabetes, in persons with chronic gastro-intestinal disturbance, or in perfectly healthy individuals who are temporarily below physical par. We have also noticed their very frequent appearance in men and women who have subjected themselves to a sudden change of physical daily habits, such as athletes going too rapidly into severe training and individuals having led a sedentary existence, living a vacation life of strenuous physical activity. Whether or not the indirect influences which determine an increased coagulability of the blood and a lessened water content in the circulation have any direct bearing upon this is doubtful, though it has been suggested by Wright. However this may be, all these factors indicate a lowered resistance in these cases which it is logical to assume could be improved by specific immunization.

It is impossible to lay down general rules for the treatment of such cases. It may be said, however, that treatment of staphylococcus skin infections should never be undertaken with stock vaccines. There is always time for the very simple procedure of isolation of the staphylococci which are directly responsible for the lesion in the individual, and the production of a vaccine. In treat-

ment with a properly prepared vaccine, the chief rule we would like to emphasize is the avoidance of any kind of a reaction, keeping the dosage below that which gives either local or systemic effects. We prefer a more frequent injection, but of smaller amounts, rarely giving more than a billion organisms in a dose. If the boils are of sufficient severity to cause severe temporary illness in the patient, a condition which is rare, treatment should be omitted, but pushed in the intervals between crops of boils and at times when the patient is in good condition. Injections of small doses can be made every third or fourth day, of the larger ones once a week. We have seen no advantage from any considerable gradual increasing of the dosage. It may be necessary to continue the treatment for a considerable period, and even when there has been no new boil for ten days or two weeks, it is advisable to add one or two further injections. Results of treatment can only be judged by clinical observation and the opsonic index is not of practical value in our opinion.

Vaccine Treatment in Gonorrheal Infections.—Here, again, autogenous vaccines should be used because, as Torrey has shown, the gonococcus group is composed of many antigenically varying members. The organisms should be cultivated from the patient whenever possible, therefore, and a homologous vaccine made.

Although the literature on the beneficial effects of gonococcus vaccine is very confusing, a few general conclusions can be drawn which, though not absolutely definite, will indicate the trend of opinion. Geraghty,[37] in summarizing the results obtained, states that he believes that there is no evidence indicating successful treatment of acute or subacute urethral infections. He states, however, that he has seen occasional excellent results in acute complications in arthritis and epididymitis. He adds that in most instances in which he has seen favorable results, he has observed acute reactions with temperature and leukocytosis, occasionally of sufficient severity to be alarming. He concludes therefore that since the vaccine is of absolutely no use in acute or chronic urethritis, and only occasionally gives symptomatic relief in the complications mentioned, the benefit derived is not proportionate to the risk incurred.

Without having any practical experience with this form of treatment, we gather from Geraghty's article, in the cases in which the severe reactions occurred, there might have been accidental intravenous or intramuscular introduction of the vaccine with the severe non-specific effects noted in such cases.

Active Immunization against Anthrax.—This method, extensively used for the protection of cattle, sheep and other domestic animals subject to anthrax, was first developed by Pasteur. The principle is the administration of anthrax bacilli which have been at-

[37] Geraghty. *Jour. A. M. A.*, Vol. 76, 1921, p. 35.

tenuated by cultivation at 42° to 43° C. in broth. The resultant strain, cultivated under these conditions for a sufficiently long time, is not only less virulent, but loses its power of spore formation. The reduction of virulence is in direct relationship to the length of time for which the cultivation is continued. The relative virulence of a given culture can be estimated by a method described by Koch, Gaffky and Loeffler.[38] Rabbits are less susceptible than guinea pigs, and virulent anthrax cultures grown for 2 to 3 days under the conditions of increased temperature, lose their power to kill rabbits in certain doses, but are still virulent for guinea pigs in these amounts. After 10 to 20 days of further cultivation at 42°, the virulence for guinea pigs disappears, but the culture is still potent for mice. Even mouse virulence may eventually be eliminated by further cultivation at this temperature.

Pasteur's procedure was to grow anthrax cultures for different periods, under these conditions. A culture which has lost its virulence for guinea pigs and rabbits, and is still potent against mice was his "premier vaccin." A culture which was still definitely virulent for mice and guinea pigs, but no longer for rabbits, was his "deuxième vaccin." Such strains in which the attenuation has been definitely determined are grown in broth for 48 hours, and are then injected into cattle in doses of 0.25 c. c., and into sheep in about one-half of this dose. The "premier vaccin" is first injected, and 12 days later the "deuxième." The immunity may last one or even two years.

A modification of Pasteur's method was that of Chauveaux[39] who grew the bacilli in bouillon at 38° to 39° C., under a pressure of eight atmospheres. The Pasteur method has been introduced all over the world, and, according to statistics gathered by Sobernheim, has had excellent results. For statistical facts on the success of the treatment, we refer the reader to Sobernheim's article in the Third Volume of Kolle and Wassermann's Handbook.

Prophylaxis against Small-pox.—In the case of small-pox the general method of active prophylactic immunization is in principle identical with that devised by Jenner in the 18th century. The original observation from which Jenner worked was that dairy maids and other individuals who had been infected with cow pox were thereafter spared when a small-pox epidemic appeared in the region in which they lived. It is now agreed by most observers who have studied the problem that the virus of cow pox and that of small-pox are identical in nature; the former representing a strain attenuated by passage through the animal body. This is based chiefly upon the observation that true variola can be transmitted to cattle, and that it can be thus carried from animal to animal, during this process

[38] Koch, Gaffky and Loeffler. *Mitt. a. d. Kais. Gesundheitsamt,* 1884.
[39] Chauveaux, *Compt. Rend. de l'Acad. Sc.,* 1884.

becoming attenuated for human beings to such a degree that reinoculated into man a simple vaccinia is produced.[40]

Small-pox, therefore, represents in principle active immunization by means of attenuated virus. When vaccination was first introduced the virus was taken from preceding pustules produced in other human beings. This has been given up in most countries to-day largely because of the dangers of transferring syphilis and other diseases in this way. At present the method of obtaining virus for vaccination purposes is carried out as follows: The initial material consists of what is known as "seed" virus, which can be obtained from spontaneous cow pox or from vaccination pustules in children, or again from pustules obtained in calves after several passages of small-pox virus through these calves. From such seed virus calves may be inoculated for vaccine production or else the calves may be inoculated from the material obtained from other calves in the usual way.

Healthy young animals are used; they are washed along the abdomen, strapped down upon specially prepared tables, and the abdominal skin thoroughly cleansed with soap and water. The exact procedure varies in different places; often the skin is thoroughly cleansed with carbolic solution, and this is thoroughly removed with sterile water before inoculation, or else cleansing is relied upon without the use of germicides. Over the clean area longitudinal scratches 1 to 2 c. c. apart are made, and into these the seed virus is rubbed. The animals are then kept in a clean stall, preferably over asphalt floors, and rigid cleanliness is observed during the period of development of the pustules. After the 6th or 7th day, when the vesicles are beginning to appear, the abdomen is well washed and cleansed of superficial dirt without the use of an antiseptic, and the pulp removed from the lesions with a curette. The pulp so removed is placed into 60 per cent. glycerin and thoroughly ground up in a specially constructed mill. According to Rosenau, the animal should always be killed before the vesicles are removed, not only for humane reasons, since the same object might be attained with anesthesia, but because a thorough autopsy can then be performed to determine the health of the calf.

Vaccines so obtained always contain bacteria, the glycerin therefore serving a double purpose: one, the preservation of the virus, the other a gradual destruction of the bacteria. Rosenau has shown that the addition of 2 to 4 parts of 60 per cent. glycerin to one part weight of the pulp prevents the growth of bacteria and probably destroys them by dehydration. Most of the bacteria are destroyed within one month at 20° C. During this period, then, from 4 to 6 weeks, the glycerinated virus should not be used, and should from time to time be controlled by cultivation. At the end of this time the lymph is ready for use.

[40] Haccius. Cited from Paul, Kraus and Levaditi, Vol. 1, p. 593.

Formerly the material for the vaccination of human beings was obtained very simply by dipping ivory splinters into the fluid of pustules, allowing this to dry, and rubbing these ivory or bone points into the exudate obtained by scratching the skin of the individual to be vaccinated. This method has practically gone out of use, and to-day the ripened glycerin pulp prepared as above is taken up in small capillary glass tubes and from these blown upon the vaccination scratch. The efficiency of vaccine virus can be tested for potency by the inoculation of the ears of rabbits before use.

That the ordinary vaccine virus ripened in glycerin is often ineffective in cases that might take if vaccinated with a more potent virus, such as the "green" or unripened vaccine, is a fact which has been forced upon our notice a number of times when soldiers of the United States Army, twice negatively vaccinated with glycerinated virus, developed takes after being vaccinated with unripened or "green" French virus. This fact makes it very desirable that further research should be carried on in the production of more potent vaccines.

Noguchi has attempted to produce a vaccine without bacterial contamination by the intratesticular inoculation of male rabbits, the pulp from the infected testicles being employed. This method has not been widespread, partly because of the small yield, and partly, perhaps, because of the claim that such virus is not as potent for human beings, as a general rule, as vaccine derived from calves. As to this point, however, we have not sufficient evidence.

The method of vaccination is an important factor in success. Wright [41] has attempted the intracutaneous injection of 0.1 c. c. of a 50 per cent. diluted ordinary glycerinated virus. He obtained 70 per cent. successful takes in 227 negro soldiers by this method, when only slightly over 8 per cent. were obtained on the same cases by the incision method.

It is our opinion that one of the most important factors in successful vaccination is to completely avoid the drawing of blood. The testing of the potency of vaccine virus can be carried out on the shaven skins of rabbits, and in performing such tests in our laboratory, Frederic Parker, Jr., found that a very marked take, amounting almost to an eruption, could be obtained on one side of the abdomen of a rabbit in which the virus had merely been rubbed into the shaven skin, while a very much more moderate take with a larger separation of papules resulted on the other side where scarification with slight bleeding had been practiced.

Within slightly over two weeks after vaccination, it has been found by Kinyoun that the blood serum of the vaccinated individuals will neutralize vaccine virus if allowed to stand with it in a test tube overnight.

[41] Wright. *Jour. A. M. A.*, Vol. 71, 1918, p. 654.

In judging of the efficiency of vaccination, we may draw upon a very convincing and voluminous literature. The statistical studies of Jurgensen in Sweden show that in the pre-vaccinal period from 1774 to 1801, there were 2,050 deaths of small-pox per 1,000,000 inhabitants.

In the transitional period of nine years from 1801 to 1810, there were 680 per million. In 1810, the practice became generally employed, although it was not enforced until 1816, but in the 35 years between 1810 and 1855, there were only 169 deaths.

These statistical studies brought together, show that in the pre-vaccinal period there was a death rate of 20 per thousand, whereas, in the vaccinal period the death rate was 0.17 per thousand.

There is no question about the fact that the simple procedure of systematic vaccination will control small-pox, but it must be remembered that this implies re-vaccination at least every 7 to 10 years, and that a negative vaccination should be followed in the same individual by a second attempt about two weeks later.

Active Prophylactic Immunization in Rabies (Hydrophobia.)—Although many modifications have been suggested and actually used in different parts of the world, the most common method of immunizing against rabies still remains that originally devised by Pasteur. The Pasteur treatment takes advantage of the prolonged incubation period of rabies and is planned to confer immunity between the time of inoculation and the time at which the disease would naturally appear. Since this period in ordinary street infection by dog bite is usually 40 days or more, a considerable interval for active immunization is available. Formerly much of this time was lost in that the diagnosis of hydrophobia in the dog or other animal that had caused the injury could not be made with certainty until the results of rabbit inoculations had been obtained. Nowadays the ease with which a diagnosis of hydrophobia can be made within a few minutes by finding negri bodies in the hippocampal and cerebellar cells has added considerably to safety in that it has made possible a gain of almost two weeks in determining whether treatment should be instituted or not.

Here again, although the infectious agent of rabies is not known with certainty even at the present day, the method of Pasteur depends upon active immunization by means of an attenuated virus.

In standardizing the virus for the purpose of treatment Pasteur first produced what he calls the "virus fixe." This consists of the ordinary street virus as obtained from rabid animals passed through a considerable series of rabbits (20-30) until its virulence for these animals has reached a maximum. After a sufficient number of such rabbit passages the incubation time after intracerebral inoculation is reduced to 7 or 8 days, but can no longer be shortened by further passage. The brain and cord material of rabbits dead of rabies after

such repeated passages constitutes virus fixe. This can be preserved for considerable periods in 60 per cent. glycerin, and this is the initial material from which the attenuated preparations for treatment are produced.

In preparing the material for treatment a small amount of virus fixe is injected subdurally into rabbits, about 0.2 c. c. of a salt solution emulsion being given. The inoculation is very easily made through a small trephine opening in the skull, and contamination is very easily avoided. Just before the rabbit dies when completely paralyzed it is killed by chloroform and the cord is removed best by the method of Oschida.[42] The rabbit is nailed to a board, back uppermost, and washed with a weak antiseptic; a longitudinal incision is then made along the backbone from the occiput to the lumbar region, and the vertebral column laid bare.

After searing the tissues around the back of the head the spine is cut across just behind the occiput, and again in the same way just above the sacrum. The neck and lumbar regions are dissected loose from the skin and gauze is inserted under it to avoid contamination. The assistant grasps the end of the spinal cord as it appears in the cervical region and pulls on it very slightly while the operator with a glass rod or a piece of wire pushes against it from below. If this is carefully done the spinal nerves are torn and the cord can be gradually pulled out of place. This procedure is by far the best, although it requires a certain amount of practice.

The cords so removed are hung up by a thread in bottles containing sticks of caustic potash and exposed in a dark place to 22° to 23° C. Under these conditions of drying and temperature the virus is gradually attenuated until at the end of 13 days or more the virulence is practically nil. If removed from the drying bottles at any time during the process and kept in a refrigerator in sterile glycerin the virulence, whatever it may be at the time of placing into the glycerin, remains fairly constant for long periods. When any of this material is used for treatment little pieces of the cord ⅛ cm. in length are cut off and emulsified in 2.5 c. c. of salt solution, and this emulsion is used for injection.[43]

When patients are to be treated the principle of the treatment is to inoculate them first with cords that have been dried for considerable periods, gradually proceeding toward those that have been dried for less prolonged times and are therefore more virulent. The treatment is varied in the individual case according to the severity of the injury. Formerly treatment was begun with cords dried as long as 16 days. More recently it has been found that cords dried for longer than 8 days are practically non-virulent and correspondingly lack

[42] Oschida. *Centralbl. f. Bakt.*, Vol. 29, 1901.

[43] In our description of the methods of drying rabies, for the sake of adhering to a standard, we follow closely the directions laid down by A. M.

in antigenic value. They are no longer employed, therefore, since their use is regarded as a waste of time. The following tables, on

Scheme for Mild Treatment

Day	Cord (injections)	Amount injected			Day	Cord (injections)	Amount injected		
		Adult (c. c.)	5–10 yrs. (c. c.)	1–5 yrs. (c. c.)			Adult (c. c.)	5–10 yrs. (c. c.)	1–5 yrs. (c. c.)
1	8–7–6=3	2.5	2.5	2.0	12	4=1	2.5	2.5	2.5
2	5–4=2	2.5	2.5	1.5	13	4=1	2.5	2.5	2.5
3	4–3=2	2.5	2.5	2.0	14	3=1	2.5	2.5	2.0
4	5=1	2.5	2.5	2.5	15	3=1	2.5	2.5	2.0
5	4=1	2.5	2.5	2.5	16	2=1	2.5	2.0	1.5
6	3=1	2.5	2.5	2.0	17	2=1	2.5	2.0	1.5
7	3=1	2.5	2.5	2.0	18	4=1	2.5	2.5	2.5
8	2=1	2.5	1.5	1.0	19	3=1	2.5	2.5	2.5
9	2=1	2.5	2.0	1.5	20	2=1	2.5	2.5	2.0
10	5=1	2.5	2.5	2.5	21	2=1	2.5	2.5	2.0
11	5=1	2.5	2.5	2.5					

Scheme for Intensive Treatment

Day	Cord (injections)	Amount injected			Day	Cord (injections)	Amount injected		
		Adult (c. c.)	5–10 yrs. (c. c.)	1–5 yrs. (c. c.)			Adult (c. c.)	5–10 yrs. (c. c.)	1–5 yrs. (c.c.)
1	8–7–6=3	2.5	2.5	2.5	12	3=1	2.5	2.5	2.0
2	4–3=2	2.5	2.5	2.0	13	3=1	2.5	2.5	2.0
3	5–4=2	2.5	2.5	2.5	14	2=1	2.5	2.5	2.0
4	3=1	2.5	2.5	2.0	15	2=1	2.5	2.5	2.0
5	3=1	2.5	2.5	2.0	16	4=1	2.5	2.5	2.5
6	2=1	2.5	2.0	1.5	17	3=1	2.5	2.5	2.5
7	2=1	2.5	2.5	2.0	18	2=1	2.5	2.5	2.0
8	1=1	2.5	1.5	1.0	19	3=1	2.5	2.5	2.0
9	5=1	2.5	2.5	2.5	20	2=1	2.5	2.5	2.5
10	4=1	2.5	2.5	2.5	21	1=1	2.5	2.5	2.0
11	4=1	2.5	2.5	2.5					

Stimson, in the *U. S. P. H. S. Bull.* 65, 1910. There are various modifications used in different countries, in many cases unimportant, and it seems well to adhere to the U. S. regulations as a standard for this country.

p. 600, taken from Stimson's article in Bulletin 65 of the Hygienic Laboratory of the U. S. Public Health Service, give the standard methods of treatment as recommended by the United States Public Health Service.

This is the standard treatment used almost everywhere in the world at present.

Recently, there has been a tendency to begin with cords that have

been dried for a less prolonged period. The following scheme of treatment is taken from an article by Stimson of the United States Public Health Service.[44]

Treatment for Adult *

Day of Treatment	Cord, Dried, Days	Amount of Cord Cm.	Day of Treatment	Cord, Dried, Days	Amount of Cord Cm.
First	6	1	Twelfth	3	0.5
Second	5	1	Thirteenth	3	0.5
Third	4	1	Fourteenth	2	0.5
Fourth	3	0.5	Fifteenth	2	0.5
Fifth	3	0.5	Sixteenth	4	0.5
Sixth	2	0.5	Seventeenth	3	0.5
Seventh	2	0.5	Eighteenth	2	0.5
Eighth	1	0.5	Nineteenth	3	0.5
Ninth	5	0.5	Twentieth	2	0.5
Tenth	4	0.5	Twenty-first	1	0.5
Eleventh	4	0.5			

* Cited from Stimson. *Jour. A M. A.*, Vol. 76, 1921, p. 241.

Other methods have been recommended. One of these is that of Högyes, in which virus fixe unattenuated is used in dilution. Högyes begins by injecting 3 c. c. of a 1 to 10,000 dilution of virus fixe, gradually proceeding within 14 days to 1 c. c. of a 1 to 100 dilution.

Fixed virus attenuated by the addition of antirabic serum and chemical disinfectants (carbolic acid) and by partial digestion in gastric juice has also been used, but none of these methods has attained widespread application.

A further method used in laboratories is that of Harris and Shackell.[45] Harris [46] believes that the attenuation of rabic virus by the Pasteur method depends primarily upon the method of extracting the water. The slow desiccation of the Pasteur procedure, he thinks, acts by a gradual concentration of the salts in the brain, the action being essentially a chemical one. He has altered the method by rapidly drying the cord at 0° in a vacuum jar over sulphuric acid. Virus so dried, will retain its virulence for as long as four months if guarded against moisture. Of this he emulsifies 10 mg. in 10 c. c. of salt solution, that is one milligram to each cubic centimeter. He then tests virulence by injecting 0.1 c. c. of the dilution into the brain of a rabbit, passing his needle into the lateral ventricle so that none of the material may escape. By this method he finds that the material after three weeks' preservation may be equivalent in virulence to fresh cord. After 50 days it is 25 per cent. more infective than by the old method. After 200 days its infec-

[44] Stimson. Cited from the *Jour. A. M. A.*, Vol. 76, 1921, p. 241.
[45] Harris and Shackell. *Jour. Inf. Dis.*, Vol. 8, 1911, p. 47.
[46] Harris. *Jour. Inf. Dis.*, Vol. 13, 1913, p. 155.

tivity is equal to that of the two day cord of the old method, and after 500 days it is two and one-half times as infective as the three day cord. Harris begins his treatment with material which is about six months old. As the treatment continues, he gradually increases until he uses material which contains 100 minimal infectious doses per milligram. He controls his treatment by the preparation of charts on which he tabulates the proportion of infectious material to non-infectious material in a brain after preservation for various periods. For further particulars we refer the reader to Harris's publications.

It should be remembered that rabies treatment is a serious undertaking which should be carried out with a complete knowledge of the procedure and with the advice of laboratory workers trained in the preparation of the material.

The treatment is severe and in its course, after the first few injections, large blotches of local hypersensitiveness may appear at the sites of injection, and the patient's general health may suffer. The nervous strain, as well as the physical discomfort, may bring further severe nervous reactions.

In the extermination of rabies as a menace to human beings, recent attempts have been made to develop a method of prophylactic immunization of dogs. Rabies it must be remembered is in many parts of the world sufficiently common to make it a public health problem, and in southern countries, particularly, seems to be rather increasing than diminishing. During the war there was a considerable increase of the disease in Japan, and Umeno and Doi [47] attempted to introduce the prophylactic treatment of dogs by methods in principle the same as those used in human beings. They showed that with a single injection of virus, it is possible to immunize a dog sufficiently to protect him for one year against infection by the bites of other dogs. According to Eichhorn and Lyon [48] from whom we take this account, their method consisted of the injection of a single dose of mixed virus treated with phenol. The brain of a rabbit which has died from fixed virus was ground up in four times its volume of a mixture of 60 parts of glycerin and 40 parts of water containing 1.25 per cent. phenol. This was stored at 22° C. for two weeks, or in the icebox for 30 days to reduce its virulence. At first it was given in dilution, but later in the original condition, that is, dilution of the brain 1 in 5. After considerable preliminary work, they determined that one injection of 5 c. c. per 15 kilogram weight, could be used, and in puppies of 4 and 1/2 kilograms or less, one-half the dose should be given. Up to 1921, over 31,000 dogs were vaccinated, and in only one case did the immunity fail. Eichhorn and Lyon in 1922 reinvestigated this problem, and confirmed the findings of the Japanese investigators. In a rather small but fairly

[47] Umeno and Doi. *Kitasato. Archiv. Exp. Med.*, Vol. 4, No. 2, p. 89.
[48] Eichhorn and Lyon. *Jour. Amer. Vet. Med. Assn.*, April, 1922, p. 1.

conclusive series, they injected one dose of 5 c. c. of the virus, as recommended by the Japanese investigators, subcutaneously, and subsequently injected street virus into the anterior chamber into these dogs and into three controls. None of the vaccinated dogs died of rabies.

The method seems to us of great importance if consistently followed among pet dogs and reënforced by the proper control of stray animals. By these methods, there seems no reason to doubt that rabies can be eventually completely exterminated.

Immunity in Syphilis.—Until relatively recent years there seems to have been little question in the mind of clinicians regarding the existence of true immunity in syphilis. It was stated uncompromisingly by Ricord [49] that "an individual who had once acquired syphilis was thereafter protected against reinfection." This opinion acquired wide acceptance and was shared by most of his contemporaries. Bäumler [50] in 1875 summarizing the authoritative opinions of that period stated that "One who has once had small-pox, scarlet fever, typhus, etc., is, as a rule, not liable to these diseases again for the rest of his life. The same is true of syphilis." However, even at the time Bäumler wrote this, exceptions to the supposed rule were accumulating and he appends, to the positive statement given above, references to observed instances of second infection reported by Bidenkap,[51] H. Lee,[52] Diday,[53] Köber,[54] Zeissl,[55] and others.

The conception of the existence of a true acquired immunity was also expressed and widely accepted in the so-called "laws" of Colles and Profeta. The former, first enunciated by Beaumès and later, in 1837, stated by Abraham Colles,[56] of Dublin, is the well-known generalization based on the observation that mothers who have borne syphilitic infants were not infected by their children while suckling them, although such children might often infect wet nurses. Profeta's [57] observation was the converse of this, namely, that children born of mothers who suffered from active syphilis during the period of conception did not acquire the disease from their mothers.

Both of these phenomena appear too well founded in clinical observation to be questioned, at least as frequent occurrences. More-

[49] Ricord. *Recherches sur le Chancre*, Paris, 1858, cited from Bäumler.

[50] Bäumler. *Ziemssen's Cyclop. of Pract. Med.*, Amer. Ed., Wm. Wood and Co., N. Y., 1875, iii.

[51] Bidenkap. *Wien. med. Woch.*, 1865, cited from Bäumler.

[52] Lee, H. Lecture on Syphilis, London, 1863.

[53] Diday. *Arch. génér.*, 1862, ii, cited from Bäumler.

[54] Köber. *Berl. klin. Woch.*, 1872, No. 46.

[55] Zeissl. *Zeitschr. d. K. K. Gesell. d. Aertze in Wien*, 1858.

[56] Colles. Cited from Osler and Churchman, "Syphilis," Osler's System of Medicine.

[57] Profeta. Cited from Osler and Churchmann, "Syphilis." *Trattato Practico delle Malattie Veneree*, Palermo, 1888.

over, considering the intimate contact between mother and infant during the first months after birth, they acquire unusual importance. However, as we shall see, they have been deprived of much of their bearing as proofs of true acquired or inherited immunity by serological investigations such as those of Bauer,[58] Knöpfelmacher,[59] and others, which have shown that mothers of syphilitic children usually give positive Wassermann reactions, a fact which makes it seem likely that such women are suffering from syphilis in a latent form and are not immune in the ordinary sense. This indeed was the interpretation given to the "laws" of Colles and Profeta by Fournier [60] and by Matzenauer [61] at a time prior to that at which serological data were available. It is still unclear why such mothers should so frequently exhibit the disease in a latent form. However, this is a matter for the intelligent discussion of which we are not at the present time in the possession of sufficient information.

In the years just preceding the period of experimental investigation of syphilis upon animals much purely clinical research was carried out on the problem of reinoculation and what is commonly known as "superinfection" of syphilitic human beings. Much of this work is unavailable as scientific evidence owing to the difficulties of distinguishing, at that time, between the true chancre and the chancroid, but a considerable number of the observations then made have been of much value in pointing out directions of later research upon animals. The work has been so thoroughly analyzed both by Neisser and by Levaditi that it would be needless repetition to do so again. The records include both accidentally occurring superinfections, and purposeful experimental reinoculations. Without, therefore, going into details concerning the individual cases we may summarize the conclusions justified from a study of these observations.

1. The reports of Lasch,[62] Jadassohn,[63] Sabearéanu,[64] Queyrat,[65] Taylor,[66] H. Lee, Knowles,[67] and many others, have shown that patients are susceptible to a second inoculation during the first incubation time, that is, during the period elapsing between the first infection with syphilis and the appearance of the chancre. Second positive inoculations have also been successful at periods shortly subsequent to the appearance of the primary sore.

[58] Bauer. *Wien. klin. Woch.*, 1908, No. 28.

[59] Knöpfelmacher and Lehndorff. *Med. klin.*, 1909, No. 40; *Wien. med. Woch.*, 1909, No. 38.

[60] Fournier. *L'Hérédité Syphilitique*, Paris, 1890.

[61] Matzenauer. *Vererbung d. Syphilis*, Wien, 1905, cited from Bruck.

[62] Lasch. *Arch. f. Dermat. u. Syph.*, 1891, p. 61.

[63] Jadassohn. *Arch. f. Dermat. u. Syph.*, 1907, p. 86; *Festschr. f. Neisser*, 1907.

[64] Sabearéanu. *Thèse de Paris*, 1905, *Chancres Syph. Successif.*

[65] Queyrat. *Bull. de la Soc. Méd. des Hôp.*, 1904, No. 28, p. 905.

[66] Taylor. *Jour. Cutan. Dis.*, December, 1890.

[67] Knowles. *N. Y. Med. Jour.*, December, 1906.

Autoinoculations and reinoculations undertaken after the chancre has become well developed have been, in the main, negative, though Queyrat reports a case successfully inoculated daily up to the eleventh, and Taylor one inoculated on the fourteenth day after the appearance of the primary induration. In contrast to these and other successful attempts, many observers record failure. However, the point is not one of particular importance, since, after all, the appearance of the chancre does not mark off any fundamental change in the progressive pathological development of the disease, and indicates only the completed reaction at the point of entrance. The fact remains that analysis has revealed that reinoculation, with the appearance of the second initial lesion, is possible up to about the twentieth to the thirtieth day after the first infection with the treponemata (Mauriac [68] and Neisser state the twenty-second day, Queyrat cites a case eleven days, Linderman one twenty-four days after the appearance of the chancre). It is claimed by some of the observers, however, that even when reinoculation during this period is successful, the incubation of the second and subsequent lesions is shorter, that the induration itself is less severe, may not ulcerate, and heals more readily than the first.

In judging of the success or failure of reinoculation practised during the later days of the period above referred to, the possibility must be borne in mind that the trauma produced at the inoculation might have served to favor the development of a localized focus, the treponemata at this time being very probably well distributed throughout the body. Unsuccessful control inoculations with non-syphilitic materials in Queyrat's cases would tend to eliminate this possibility, whereas lesions resulting at the sites of such control abrasions in the experiments of Neuman and Cehak,[69] and of Levaditi,[70] appear to support it. However, this is not of great importance inasmuch as it determines merely a relative lengthening or shortening of the period during which reinoculation or superinfection is possible.

2. After the disease is well established as a systemic infection, that is, from the time of development of the chancre throughout the so-called active "secondary" period, reinoculation is either impossible or, at any rate, extremely difficult. Neisser cites Rollet as follows:

"Although I and my predecessors have a thousand times attempted to reinoculate luetic subjects, we have never observed a successful case. I know no single fact more thoroughly proven than the insusceptibility of a syphilitic to the action of a new virus, and,

[68] Mauriac. Cited from Neisser, *loc. cit.*

[69] Neuman and Cehac. *Wien. med. Bl.*, 1890, cited from Neisser.

[70] Levaditi. *De l'Immunité acquise dans la Syphilis. Zeitschr. f. Imm. Ref.*, pp. 191, 277-318.

moreover, these experiments are so harmless that they may be performed without scruple."

The same opinion was held by Mauriac and is in a general way assented to by Neisser in his summary of this place of his studies. (Neisser, *loc. cit.*, pp. 180-181.)

That the patient with well-developed lues has acquired a considerable degree of resistance to fresh inoculations is pretty generally accepted, therefore, by all who have studied the question—but there are investigators, notably Finger and Landsteiner, who believe this resistance to be less absolute than stated by earlier writers. Their observations on patients with active syphilis seem to indicate that superinfection is possible "under certain circumstances in all stages of the disease," but "the positive effect can be obtained only with considerable quantities of the virus." Furthermore, Landsteiner [71] states that, in general, lesions so obtained are relatively slight in severity, do not appear as primary indurations, but have the tendency to simulate the particular variety of lesion spontaneously manifest in the individual at the time. Were it not for Finger and Landsteiner's monkey experiments, to which we will refer directly, and which seem to bear them out in their interpretation of these inoculations as "positive" results, the simulation of the spontaneously occurring lesions by the inoculation-products would again justify suspicion that these experimental results represented merely traumata in which, as points of less resistance, the patient's pre-existent disease had found a favorable spot for localization.

Observations in this respect, also, are corroborated by the older literature. A report which has direct bearing on this point, is one of Queyrat and Pinard who inoculated a tertiary patient with chancre material, obtaining not a primary sore but an ulcerated lesion having the clinical characteristics typical of the late skin manifestations of the disease. The autoinoculation experiments of Ehrmann [72] also would tend to show that the resistance of the luetic subject is a relative one only. Ehrmann succeeded in producing positive autoinoculation-products in 45 syphilitics with papular eruptions. Control inoculations with sterile water were negative. Although his observations and those of Finger and Landsteiner, with other similar ones, teach us that superinfection during the disease is possible the nature of the lesions, their short incubation time, and their exceptional character when averaged with the total of such attempts, prevent them from invalidating the conclusion that there is a resistance at this stage higher than that of the normal subject.

We may conclude, therefore, concerning the secondary period of the disease, that the luetic individual has acquired a resistance which while not absolute is at least very high, and protects him from fresh

[71] Landsteiner. *Centralbl. f. Bakt.*, Ref. 1908, Vol. 41, p. 785.
[72] Ehrmann. Cited from Neisser, *loc. cit.*

external inoculation, although at the time his disease may still be progressing and in no sense overcome.

3. During the late stage of syphilis, the stage at which, according to the more or less arbitrary divisions of Ricord, we are accustomed to speak of it as "tertiary," the resistance is still manifest, though apparently not so regularly potent as during the preceding "secondary" period. Neisser expresses himself with great caution and accepts few, if any, of the observed reinoculations of tertiary cases as surely representing unquestionable freshly acquired infections. Nevertheless, taking into consideration a detailed study of individual reports, he concludes that resistance during the late stages is pronounced but already beginning to wane. He himself in the Festschrift to F. Joseph Pick in 1898 cites a case of the development of a chancre in a tertiary case which, however, was not followed by constitutional symptoms.

4. In the preceding paragraphs we have concerned ourselves entirely with questions of "superinfection," that is, the implantation of the syphilitic virus into subjects still suffering from manifestations of the disease. A problem of equal theoretical, and of much greater practical importance, is that dealing with true "reinfection." By "immunity" in the ordinary sense, we mean an increased resistance to specific infection which persists for a more or less prolonged period after the active disease has been overcome and the causative agents removed from the body. It is not easy to draw conclusions concerning this point from observations on human beings, since it is most difficult to decide, even with the aid of serological methods, whether or not a given case is cured in the bacteriological sense; for syphilis is preëminently the disease in which there occur frequent and prolonged latent periods terminated, often after lapses of years, by the reappearance of foci of a grave nature.

Moreover, our own experiments both with syphilis in rabbits and the treponemata in culture have convinced us that these micro-organisms may assume for long periods a condition of metabolic latency, a sort of resting period, during which they incite no reactions of any kind on the part of the tissues, apparently do not multiply to any great extent and yet remain alive and capable of development when conditions favor this.

In spite of these difficulties, however, careful clinical studies by Jonathan Hutchinson, Taylor, Hudélo,[73] Neisser, and many others, have furnished data which warrant an opinion upon this problem. Of especial value is the painstaking analysis of reported cases of reinfection in syphilis made in 1909 by Felix John.[74]

In contrast with some others who have attempted similar analyses

[73] Hudélo. *Ann. de dermat. et de syph.*, 1890, p. 353.

[74] John, Felix. *Reinfectio Syphilitica, Samml. klin. Vortr., Volkmann,* 1907-1909, p. 559 *et seq.*

John takes the utmost care to separate these cases into the ones in which the evidence of true reinfection is absolute, and those in which the reported details are insufficient to exclude the possibility of recrudescence. In agreement with Taylor he insists upon a symptom-free interval of five years between the last manifestations of the first attack and the appearance of the second. As an example of what he calls an "Ideal Fall" we may cite the following:

X. J., April 1, 1872, primary sore.

Roseola, polyadenitis, mucous patches. September, 1872, papular rash. March, 1873, palmar and plantar spots, iritis. 1875, gumma of tibia and serpiginous syphilide of right thigh.

Four courses of inunctions and KI.

1876, married—two healthy children.

No symptoms till 1887 when he acquired a second chancre followed by typical roseola. In 1888 wife had still-birth.

John has analyzed in this way 356 cases of supposed reinfection, in 34 of which the first attack was of congenital origin. Of the remaining 322, fourteen were cases which seemed unquestionable instances of reinfection and in 16 more there was practically no doubt of this. Of the 34 hereditary cases there were three, one of Emery, one of Taylor, and one of Hochsinger, in which there was practically no question of their nature as valid reinfections. In all of the others there was one point or another which rendered them doubtful as evidence.

John concludes that true reinfection can unquestionably occur in syphilis but that it is relatively rare.

To John's cases Neisser has added others reported between 1909 and 1911. Yet even with these, the total number is not a large one Nevertheless, we should not be tempted to conclude from this that the relative infrequency of such cases is evidence in favor of the existence of a true immunity analogous to that following typhoid, plague, etc. For we have seen that a very definite insusceptibility is coincident with the persistence of actual disease in the subject and, as Neisser points out, the more recent investigations carried out with the aid of serological tests have shown that the number of cases uncured though long without symptoms, is much larger than formerly supposed. The scarcity of true reinfection, therefore, may well be due to the relative scarcity of completely cured cases. Moreover, it must be remembered that, in this disease, even when final recovery results, it is usually achieved only at an age when the individual is less exposed to reinfection because of changed economic and domestic conditions, or by reason of the virtue which comes with arteriosclerosis and the belated wisdom of the burnt child that fears the fire.

Granted, then, that true reinfection is possible, is there any evidence that when it does occur, the second attack is less severe and

more easily cured than the first, a fact which would also tend to support the opinion that a certain degree of immunity persists. Jonathan Hutchinson [75] expressed this view in a clinical lecture in which he states that "second chancres are far more common than second attacks of constitutional syphilis." However, John in his summary, in which Hutchinson's cases are included, finds that in general the disease has run a second course very similar as to severity to that incident to the first infection.

If we gather together, then, the facts revealed by clinical study we may conclude with Levaditi, Neisser, and most others, that:

1. The syphilitic subject acquires definite resistance to reinoculation which becomes manifest soon after the appearance of the primary sore, at a time when the virus may be regarded as having gained universal systemic distribution.

2. This resistance, high though not absolute, persists throughout the secondary or most active period of the disease and into the tertiary stage. During the latter, however, it appears somewhat tc decline, reinoculation or superinfection being more frequently possible at this period.

3. When syphilis is entirely cured, susceptibility may in all probability be regarded as returning, possibly, though not certainly, to the same degree as it exists in the normal subject. The reasons for this last belief will become more clear when we study the evidence contributed by animal experimentation.

Notwithstanding the admirable thoroughness with which clinical data had been collected and analyzed in the study of syphilis, progress beyond the points indicated in the preceding sections was quite impossible without the aid of animal experimentation and a knowledge of the causative agent. Fortunately these two deficiencies in our methods were removed when in 1903 Metchnikoff and Roux succeeded in transmitting the disease to a chimpanzee, and in March, 1905, Schaudinn [76] discovered the treponema pallidum.

As a matter of fact, probable transmission of syphilis to lower monkeys had been accomplished as early as 1879 by Klebs [77] and subsequently by Neumann,[78] Martineau,[79] and Charles Nicolle,[80] but in none of these experiments had it been possible to prove beyond question the syphilitic nature of the inoculation-products. In the chimpanzees inoculated by Metchnikoff and Roux, the animals developed not only primary sores but also secondary eruptions, polyadenitis, and enlarged spleens in such characteristic manner that the

[75] Hutchinson, J. *Brit. Med. Jour.*, Vol. I, 1882, p. 699.

[76] Schaudinn. *Deutsch. med. Woch.*, 1905, No. 42; *Arb. a. d. Kais. Gsndhtsamte,* Vol. 26, 1907.

[77] Klebs. *Arch. f. exper. Path. u. Pharmakol.*, 1879.

[78] Neumann. Cited from Mühlens, *loc. cit.*

[79] Martineau. *Arch. f. Dermat. u. Syph.*, 1884, No. 16.

[80] Nicolle, Ch. Cited from Mühlens, *loc. cit.*

identity of the inoculation disease with human syphilis could no longer be doubted.

Successful transmission to other anthropoids and to lower monkeys were then announced, in rapid succession, by Metchnikoff and Roux, Ch. Nicolle, Neisser, Baermann and Halberstaedter, Finger and Landsteiner, Hoffman, and others. The susceptibility of monkeys was tabulated by Neisser in the following series: Chimpanzee, Gibbon, Orang-Outang, Cynocephalus babuin, Cynocephalus sphinx, Cynocephalus hamadryas, Cercopithecus fulginosus, Macacus niger, M. nemestrinus, M. cynomolgus, M. sinicus, M. speciosus, M. rhesus. In 1906 Bertarelli [81] produced syphilitic keratitis in rabbits, demonstrating the treponema pallidum in sections of the cornea and in 1907 Parodi [82] first produced syphilitic orchitis in the same animals.

Apart from monkeys and rabbits, no animal species have so far been shown sufficiently susceptible to be available for systematic study. It is true that the production of keratitis is claimed in dogs and sheep (Bertarelli, Hoffman and Brünig), in guinea pigs (Bertarelli), in cats (Levaditi and Yamanouchi), and in goats (Bertarelli). However, these experiments have been isolated and too uncertain with present methods to offer material for experimentation. Our own attempts on cats, pigs, guinea pigs, rats, mice, and a few birds, have yielded negative results only. There are many observations of great scientific interest which might be discussed in connection with the problem of susceptibility of various animal species; however, we will confine ourselves at present to those phases of the work only which have bearing on the questions of immunity.

In the fundamental premises, the work on monkeys has pretty accurately confirmed the observations concerning reinoculation and superinfection previously made on human beings.

The most extensive studies in this respect are those of Neisser [83] and his associates in the Javan expedition. Briefly stated, Neisser reinoculated 135 animals 165 times with negative result on the second and subsequent inoculations. The second inoculations were made at periods ranging from 21 days to two years after the first. In but 27 animals did the reinoculation show positive results, and in ten of these cases only does Neisser recognize the experiments as valid. Although these ten positive reinoculations, it is true, add an element of irregularity to the series, they constitute but 6.8 per cent. of the entire number, a proportion which in no way invalidates the experiments when we consider that the work was done entirely on the lower monkeys, animals that are far less susceptible to syphilis than are human beings and in many of which, therefore, systemic distribution of the virus (a generalization apparently necessary for the

[81] Bertarelli. *Centralbl. f. Bakt.*, Orig. Vol. 41, 1906, and Vol. 43, 1907.
[82] Parodi. *Centralbl. f. Bakt.*, Orig. Vol. 44, 1907.
[83] Neisser. *Beitr. z. Pathol. u. Ther. d. Syphilis*, Springer, Berlin, 1911.

development of resistance) may not have taken place. Neisser's conclusions, therefore, that monkeys, like human beings, are not reinoculable while suffering from systemic syphilis, seem entirely justified.

His work, as well as that of Finger and Landsteiner, and of Kraus and Volk [84] on lower monkeys, has shown that resistance does not develop until the twelfth to the twentieth or twenty-first day after the first inoculation; that is, again as in man, when the virus has become generally distributed. Finger and Landsteiner, furthermore, noticed that reinoculation-products, obtained by reinfection during the first incubation period, that is, before the development of the primary lesion, were less severe and developed in a shorter time than did the first lesion. This phenomenon which would tend to mark another analogy to the conditions prevailing in human beings, was not observed in the experiments of Neisser and of Kraus and Volk. However, like the similar observation in human beings, it seems to indicate the gradual acquisition of resistance as the virus begins to exert its influence upon the tissues.

Again, with monkeys as in man, the question arises whether the resistance so unquestionably proven is a condition merely coexistent with active disease, or whether it may be interpreted as a true immunity which persists after the micro-organisms have been completely removed. The most directly pertinent experiments are those of Neisser. Neisser reinoculated monkeys at periods ranging from 27 to 645 days after the first infection. After waiting a time sufficiently long to insure the negative result of the reinoculation, he used organ-substance from these animals to inoculate other monkeys. In 22 experiments of this kind he obtained positive results—showing that the organs of the apparently immune animals still harbored virulent treponemata. In seven animals only did the inoculations with organ-substance fail to produce lesions, but of these all but one died before the 30th day after inoculation.

In contrast to these results Neisser found that animals which had been "cured" by various treponemacidal agents such as atoxyl, arsacetin, etc., were almost regularly reinoculable. In fact, these experiments were so uniform that Neisser later utilized the reinoculation method as an index of cure or persistence of the disease.

The results obtained in monkeys, therefore, are very similar to those determined by clinical observations in man, and the following statements may be taken as summarizing the conditions revealed by monkey experiment;

1. That the body develops resistance, progressively increasing as the virus becomes generally distributed.

[84] Kraus and Volk. *Wien. klin. Woch.*, 1906, No. 21, Ref. IX. *Kongress d. Dermatol. Gsell. in Bern.*

2. That the resistance probably reaches its highest development during the early tertiary period.

3. That complete cure is probably synonymous with gradual return of susceptibility.

The only other animals on which systematic experimentation has been possible up to the present time have been rabbits. Since Bertarelli's successful production of keratitis and Parodi's inoculation of the testes in these animals, they have been studied carefully by a number of workers, chiefly Uhlenhuth and Mulzer,[85] and Noguchi;[86] and in our own laboratory, with Hopkins and McBurney, the writer has observed rabbit syphilis for a number of years. From a large mass of observations it appears that the conditions in rabbits are not identical with those observed in man and monkeys. So far, the testes and the eyes are the only organs in these animals in which syphilitic lesions can regularly be produced, and although treponema pallidum may apparently be distributed generally to the organs after inoculation, it does not easily arouse pathological reactions except in the organs named, and the lesions produced are not accompanied by a generalized resistance comparable to that discussed in connection with the higher animals in preceding sections of this paper.

Bertarelli found that he could reinoculate the cornea in a rabbit that had previously been inoculated with syphilis. Uhlenhuth and Mulzer, and Neisser and Pürckhauer, showed that infections of the eye could be produced while the opposite eye was still syphilitic. Tomazewski found that scrotal infection did not protect against infection of the cornea, and *vice versa*. Furthermore, Uhlenhuth and Mulzer, on the basis of a very thorough study, believe that such reinfections are neither less extensive nor more rapid in healing than was the first lesion. The same authors noticed resistance to reinoculation in two animals only, and these were young rabbits in the first weeks of life, which, they believed, had been generally syphilized by intracardial injections. Interesting in this connection are claims by Ossola and Truffi who believe that successful skin inoculation in rabbits confers a certain amount of skin resistance and this is in harmony with the belief of Kraus and Volk, that a specific skin immunity in syphilis is possible. Of great interest to us and of a possible theoretical bearing which we will discuss below, are observations made by the writer with Hopkins and McBurney[87] on 20 rabbits which were reinoculated into the testes after apparent healing of lesions in these organs. It appeared that in rabbits the opposite

[85] Uhlenhuth and Mulzer. *Arb. a. d. k. Gsndhtsamte,* Vol. 44, 1913.

[86] Noguchi, H. *Jour. A. M. A.,* Vol. 58, 1912, p. 1163.

[87] Zinsser, H., Hopkins, J. G., and McBurney, M. *Jour. Exp. Med.,* Vol. 23, 1916, pp. 329, 341.

testis can be successfully inoculated before, during, or after, the existence of a testicular lesion on one side, but that reinoculation of the same testis which had apparently returned to normal, at periods ranging from 6 weeks to one year, was not often successful. There is a certain amount of evidence here of a purely local immunity, a matter which we will discuss at greater length presently. Perhaps the difference between rabbits and the higher animals lies chiefly in the fact that syphilis is not generalized in the same sense that it is in man and monkeys, and even though inoculations from the organs of syphilitic rabbits may often result positively, this might signify only that the treponemata have been generally distributed by the blood stream and latently lodged in the organs, without, however, arousing in the tissues any sort of pathological response. This idea would seem to be borne out by the two experiments of Uhlenhuth and Mulzer cited above in very young rabbits since in such animals true generalization seems to be more common. The study of very young rabbits will be continued with this point in view.

It is apparent from the preceding considerations that resistance to syphilis differs from that acquired in many bacterial infections chiefly in the fact that it does not persist after the disease is over, but probably coexists only with the presence of the living incitants in the body. In this respect it seems to be similar to the conditions prevailing in many protozoan diseases. Thus Schilling [88] cites a case of Trypanosoma Brucei in a steer, experimentally infected by Koch, in which reinoculation was repeatedly negative, this result being at first falsely interpreted as immunity; but six years later Kleine found that the same steer still harbored the trypanosomes in his blood—showing that the resistance to reinoculation was not, in this case, an evidence of true immunity, but rather represented a condition of insusceptibility to "superinfection" analogous in every respect to that existing in syphilis. Similar conditions have been shown to prevail in Texas fever and Schilling believes that they may be regarded as also existing to a certain extent in Malaria and Sleeping Sickness. In both of these conditions complete spontaneous sterilization of the body (without medicinal aid) is probably rare, possibly does not occur at all, and the apparent immunity to reinfection is, as in syphilis, an evidence of persistence of the disease in a latent form.

In weighing the analogy of syphilis to such protozoan diseases, one is inclined to wonder whether syphilis in man may be regarded as at all spontaneously curable. So few are the cases left untreated and so rarely does the reinfection occur even in the face of specific remedies, that it seems to us more than likely that in syphilis, as in Sleeping Sickness, a spontaneous "sterilizing" immunity does not

[88] Schilling. *In Kolle u. Wassermann Handb. d. Path. Mikroorg.*, Vol. 17, p. 600.

occur. This is a point, however, regarding which it is impossible to gather data.

In order to distinguish the conditions outlined above tersely from the ordinary conception of immunity, Neisser [89] speaks of the alterations which govern the reactions of the luetic body to freshly introduced virus as "Anergie;" and "Umstimmung" or *"Allergie."* By *"Anergie"* (a term first used by v. Pirquet and subsequently introduced by Siebert in working out the analogy between pigeon-epithelioma and syphilis), Neisser designates a condition of inability to react by cellular change to contact with the virus. As he uses it, it implies a passivity on the part of the invaded tissues (in which "die Zellen auf die Spirochaeten schwer oder garnicht reagiren), by which there is not necessarily a destruction of the invading treponemata, and which, therefore, cannot in any sense be interpreted as a "Schutz wirkung." By the "Umstimmung" of Neisser, the "changement dans la mode de reaction" of Levaditi, is meant the changed reaction capacity of the syphilized tissues which determines the characters of the lesions at various stages of the disease. Thus it is obvious that cellular reactions which result in the primary induration are quite different from those which produce the tertiary gumma, and that the histological changes of the roseola are distinct from those of the serpiginous syphilide of the late stages. And since, as we shall see, there is no valid reason to assume that the incitant has been modified in virulence or vitality, we are forced to believe that the reaction capacity of the body cells has been altered.

Since it is a fact, then, that syphilitic infection so changes the body tissues of man and monkeys that, during its course, resistance to reinfection is produced, it should be possible to analyze this resistance into its responsible factors and perhaps utilize the knowledge so gained for practical therapeutic purposes. Before we proceed to do this, it will be of advantage to review briefly the attempts at active and passive immunization which have been made in animals and man.

Metchnikoff and Roux [90] followed their first animal inoculation studies by extensive vaccination experiments. Some of their first work along these lines is perhaps marred to some extent by an insufficient recognition of the resistance which depends upon persistence of the disease rather than upon true immunity, but a good many of their observations are of fundamental importance. It will be convenient to classify their work and that of others into experiments dealing with "active" and those dealing with "passive" immunization.

[89] Neisser. *Baermann u. Halberstaedfer d. med. Woch.*, 1906, Nos. 1 and 3.

[90] Metchnikoff and Roux. *Ann. de l'Inst. Past.*, Vol. 17, 1903, p. 809; Vol. 18, 1904, pp. 1, 605; Vol, 19, 1905, p. 673; Vol. 20, 1906, p. 785.

Metchnikoff and Roux first worked with filtered virus and virus killed at 51° C. All their attempts with such material were negative in that the monkeys treated with it could not be regarded in any sense as immunized. In their reports of these experiments they remark that they believed this to be due to an absolute loss of power to incite reaction on the part of the vaccine-material. We emphasize this point here because our own subsequent work inclines us to believe, with them, that the production of a reaction is necessary for the development of any considerable degree of resistance.

Neisser carried out a large number of attempts at vaccination in which he used extracts of syphilitic primary lesions and of the organs of congenitally syphilitic children, killed by the addition of carbolic acid. Unfortunately he assumed that his extract contained syphilitic antigen because it gave positive reactions by the complement fixation technique of Wassermann, an assumption which we of course know now to be unfounded as far as any relation to the body substance of the treponemata is concerned. This, to our mind, deprives these particular experiments entirely of their negative importance.

In rabbits a large amount of work has been done by Uhlenhuth and Mulzer. They injected living material from rabbit lesions intravenously and subcutaneously, without ever observing any evidence of protection against subsequent inoculations.

Of perhaps the greatest importance in connection with active immunization are the attempts made upon human beings by different investigators.

Casagrandi and De Luca [91] tried prophylactic immunization on six human beings by injection of filtrates obtained from primary lesions. Two of these people later contracted syphilis in the ordinary way.

Possibly the most hopeful results are those obtained by Spitzer [92] by a method suggested by Kraus. Kraus,[93] reasoning from the fact that syphilis like hydrophobia was a disease with long incubation time, expressed the hope that perhaps the method of Pasteur in hydrophobia, that is, active immunization during the period of incubation, might, in syphilis also, tend to abort the disease. Accordingly, Spitzer treated 15 cases of early syphilis immediately after the appearance of the chancre by subcutaneous injections of emulsions made from human chancre material in dilutions of 1:200 to 1:20. The cases received from 11 to 20 injections and in seven of them the disease was uninfluenced. In the others, however, subsequent symptoms were delayed, and in four, no generalized symptoms occurred. In a later communication, Spitzer reported 23 further cases similarly treated, 10 of which failed to develop generalized

[91] Casagrandi and de Luca. *Gior. ital. de mal. ven.*, 1905.
[92] Spitzer. *Wien. klin. Woch.*, 1905, 45, and 1906, 38.
[93] Kraus. *Wien. klin. Woch.*, 1905, No. 41, and 1906, No. 21.

symptoms and in 9 of these the Wassermann test remained negative. One of them, a fact which is of great importance, was spontaneously reinfected with syphilis two and one-half years later. These results, if accurate in every way, are of the greatest importance, but are diametrically opposed to the experience of all other investigators. Monkey experiments along the same lines by Neisser gave entirely negative results and Brandweiner [94] as well as Kreibich [95] were unable to confirm Spitzer's results in man. Further comment on the Kraus and Spitzer method is valueless without more experimental data. It is one of the few rays of hope, but so isolated that one is forced to skepticism. Metchnikoff and Roux did almost the identical thing in an orang-outang and obtained lesions both at the point of the original inoculation as well as that at which the subsequent "protective" injection was made.

The only experiments in which an attempt at vaccination with *attenuated virus* was made with some indication of efficacy, is one of Metchnikoff and Roux, the outcome of an accidental laboratory infection. It appears that a laboratory assistant who had been attending to the animals, noticed a small lesion on his lip which did not look like a typical syphilitic chancre. In order to allay the patient's fears, however, Metchnikoff and Roux did inoculations from the patient to monkeys and these were positive. Nevertheless, Fournier after examining the original lesion declared it so unlike the ordinary primary sore that he did not advise treatment. No secondaries developed in the patient nor in the three chimpanzees inoculated with the material. From this occurrence Metchnikoff and Roux concluded that the patient had probably been infected in handling the monkeys, and that the virus had become attenuated by passage through these animals. On the basis of this observation they later inoculated a willing subject 79 years old with virus carried for five generations in lower monkeys. The lesion which developed was very slight, consisting only of a local induration, and no generalized symptoms developed. A previous attack of syphilis Metchnikoff believes could be reliably excluded in the subject. The experimenters suggest that the passage through lower monkeys may attenuate the virus for man, this leading to relative immunity to subsequent inoculations, and furnish a possible means of protection. Their experiments are too few to permit conclusions as yet, but even should they hold good, the method would seem to imply a considerable degree of risk and our experience with superinfections and reinfections in syphilis does not encourage the hope that a method so drastic would be justified when the benefit to the patient is apt to be of such duration only.

The history of passive immunization is an extensive one and hardly worth going into with any degree of detail since many times

[94] Brandweiner. *Wien. med. Woch.*, 1905, No. 45.
[95] Kreibich. *Wien. klin. Woch.*, 1906, No. 8.

extravagant claims have been made only to be refuted by accurate study. There is a great similarity in respect to this between syphilis studies and those in tuberculosis and cancer. A brief examination of the bibliography in Neisser's book is sufficient to convince one of the many attempts that have been made in this direction and often by methods as ludicrous as the claims of success for which they formed the basis. The most careful and skillful workers have uniformly reported failure. Metchnikoff and Roux treated various monkeys with blood from syphilitic patients and used the serum of these animals for protective experiments. There are a few instances in which mixtures of such serum with syphilitic virus rendered this inactive on inoculation. A powder made of this serum was supposed to have some protective effect when applied to fresh inoculation spots within the first hour after inoculation. However, injection of the serum had no effect whatever. Casagrandi and De Luca using serum of a dog treated with syphilitic virus, obtained entirely negative results, and Finger and Landsteiner [96] report negative results with monkey blood in man. The most extensive experiments were again those carried out by Neisser and his associates. They were done by the treatment of animals with dead and living syphilis virus, organ extracts, with the blood of syphilitic man and monkey, and horses, sheep, and monkeys were used for the production of "immune" serum. In no case was there the slightest protective effect on the part of the serum either *in vitro* or *in vivo,* and the results of the experiment were unqualifiedly negative.

When the method of complement fixation was successfully applied to the diagnosis of syphilis first by Detre, and then by Wassermann, Neisser and Bruck,[97] it was generally assumed that this reaction incidentally demonstrated that specific antibodies were formed in syphilis. As matters have developed, however, this point of view can no longer be maintained. It was soon discovered that the antigen used for these reactions by Wassermann and his associates derived its "fixing" constituent not from the body substances of the treponemata contained in the syphilitic organs, but from certain tissue extractives chiefly of lipoidal nature which could be obtained readily from normal as well as syphilitic tissues. Although Bruck [98] and others who have occupied themselves with the theoretical basis of the Wassermann reaction, still maintain that a specific antibody may be incidentally involved, they admit that this is the less important factor in the reaction which depends chiefly upon the existence of lipotropic substances which appear in the course of the

[96] Finger and Landsteiner. *Centralbl. f. Bakt.*, Ref. Vol. 38, 1906.

[97] Wassermann, A., Neisser, A., and Bruck, C. *Deutsch. med. Woch.*, Vol. 32, 1906, p. 755.

[98] Bruck. *Imm. bei Syph. in Kolle u. Wassermann Handb. d. Pathog. Mikroorg.*, Vol. 7, p. 1045 *et seq.*

disease as metabolic products either of the body or possibly of the treponemata. This is a subject which we will deal with *in extenso* in another paper. It is sufficient for our present purposes to point out that, although to a slight extent specific antibodies may play a part in the Wassermann reaction, this is certainly not the chief element or even a very important factor involved. The truth of this conception has been further confirmed by the work of Noguchi,[99] of Craig and Nichols,[100] of Kolmer,[101] and ourselves, in which it has been found that antigens made with pure cultures of treponemata produced a complement fixation with syphilitic sera to a very limited extent only, and in our work in which we have been able to duplicate the Wassermann reaction in a large series of antigens made from the treponema cultures, we have found that similar results could be obtained with cultures of colon and typhoid bacilli identically prepared, the fixing power to a large degree depending upon the lipoidal constituents of the bacteria. Whatever the ultimate explanation of the Wassermann reaction may turn out to be (and this is a subject which it would not be profitable to discuss at length at this place), it cannot be maintained that as it exists at present, it can be interpreted as demonstrating the existence of true circulating antibodies, analogous to those found in bacterial diseases in the blood of syphilitic patients.

Early in the history of such investigations, Fornet,[102] with his collaborators, Schereschewsky, Eisenzimmer, and Rosenfeld,[103] found that when the sera of syphilitics were mixed with clear extracts of syphilitic livers similar to those used in the Wassermann reactions, precipitates formed which were not seen in similar experiments done with normal sera. This Fornet [104] in a number of communications interpreted as showing the formation of precipitins in the course of syphilis. At almost the same time, L. Michaelis [105] made similar observations giving them the same interpretations. The experiments of Fornet have not found universal confirmation, but even if such precipitin reactions can be occasionally observed, they do not indicate true precipitins for the same reasons that the Wassermann reaction does not demonstrate true complement fixation of antibodies. The antigens did not represent strong treponema antigens and Jacobsthal [106] and others have shown that with the ordinary Wassermann

[99] Noguchi, H. *Jour. Exp. Med.*, Vol. 16, 1911, p. 99.
[100] Craig, C. F., and Nichols, H. J. *Jour. Exp. Med.*, Vol. 16, 1912, p. 336.
[101] Kolmer, J. A. *Jour. Exp. Med.*, Vol. 18, 1913, p. 18.
[102] Fornet. *XIV Internat. Kongress f. Hyg. und Derm.*, Sept. 1, 1907.
[103] Fornet, Schereschewsky, Eisenzimmer and Rosenfeld. *Deutsch. med. Woch.*, 1901, No. 41.
[104] Fornet, Schereschewsky. *Münch. med. Woch.*, 1907, No. 30; *Berl. klin. Woch.*, 1908, p. 85.
[105] Michaelis. *Berl. klin. Woch.*, 1907, No. 46.
[106] Jacobsthal. *Münch. med. Woch.*, Vol. 57, 1910, p. 214.

antigens syphilitic sera can produce precipitations visible under the dark field, the basis of the complement fixation being, therefore, one of probable colloidal precipitation. The formation of true precipitins, therefore, has not been shown for syphilis.

Investigations of the effect produced upon virulent treponemata by the sera of syphilitic individuals have likewise been unsatisfactory. Hoffmann and Prowazek [107] reported in 1906 that the serum of syphilitics in the later stages produced immobilization of the virulent treponema pallidum, an observation which was confirmed by Zabolotny.[108] Landsteiner and Mucha [109] were not able to observe such immobilization, nor did they see any evidence of agglutination in such experiments. In a limited observation of our own, we have also failed to see any regular or distinct influences of this kind. It is not impossible that a slight difference may exist in this respect between syphilitic and normal sera, but even if it occurs, the action is feeble, irregular, and entirely insufficient to be interpreted as having much practical importance in the acquisition of syphilis immunity.

Treponemacidal experiments have been done by some workers also with negative results. As we have stated in another part of this paper, the attempt to treat syphilitic animals and man with the serum of syphilitics has led to no reliable results, and no evidence whatever that can be accepted has been adduced to show that virulent treponemata may be killed by active luetic serum.

As far as any opsonic action is concerned, Levaditi [110] in histological studies in congenitally syphilitic children has seen phagocytosis or at least intracellular localization of treponemata in the alveolar cells of the lung and in the parenchyma cells of the liver and kidneys. For reasons not entirely clear to us, he interprets the former as true phagocytosis and the latter as a penetration of the treponemata into the liver cells to the detriment of the latter. We ourselves have occasionally seen phagocytosis in sections of syphilitic rabbit testes, but in all cases the process was not a very active one and not much can be said about its importance at present. As a matter of fact, Hopkins and the writer,[111] in studying the mechanism of the natural resistance of mice against syphilis, injected virulent organisms into the peritoneal cavities and observed the treponemata

[107] Hoffmann, E., and Prowazek, S. *Centralbl. f. Bakt.*, I. O., Vol. 41, 1906, pp. 741, 817. (Balanitis and montu spirochætæ.)

[108] Zabolotny, D., and Maslakowitz. *Centralbl. f. Bakt.*, I. O., Vol. 44, p. 532.

[109] Landsteiner and Mucha. *Wien. klin. Woch.*, Vol. 19, 1906, p. 1349; *Centralbl. f. Bakt.*, Vol. 39, 1907, I. R., p. 540.

[110] Levaditi. *Compt. rend. Soc. de biol.*, Vol. 60, 1906, p. 134; *Ann. de l'Inst. Past.*, Vol. 20, 1906, p. 41.

[111] Zinsser, H., and Hopkins, J. G. *Jour. A. M. A.*, Vol. 62, 1914, p. 1802; *Jour. Exp. Med.*, Vol. 21, 1915, p. 576.

in peritoneal puncture fluid, alive, actively motile, and unphagocyted, though surrounded by masses of leukocytes, as long as three days after their injection. It seemed almost as though the natural immunity of such animals might be similar to the "atreptic" cancer resistance spoken of by Ehrlich. The treponemata did not multiply in the mice but though, naturally, diminishing in number, were apparently neither killed nor even inhibited in motility by the peritoneal exudates and, for several days, swam in and out among the accumulating leukocytes, often adhering to them peripherally but not taken by them. Lack of entirely satisfactory methods of staining cells containing treponemata make it difficult to speak with certainty of the actual occurrences. But we gained the distinct impression that the treponemata were not actively injured or destroyed until they had spontaneously died out owing to lack of suitable environment, *i. e.,* nutrition.

In the case of natural immunity at any rate, we do not think that phagocytosis by the mobile leukocytes plays a primarily important rôle. However, these experiments will need further elaboration.

The search for antibodies gained new vigor when the efforts of Schereschewsky,[112] Mühlens,[113] Hoffmann,[114] and especially Noguchi, had resulted in successful cultivation of treponemata from syphilitic lesions in man and animals. It was hoped that with the causative organisms isolated, immunization and a clear understanding of the antibodies in syphilis might yield practical results. Kolmer [115] observed that cultivated treponemata were agglutinated in the sera of rabbits treated with culture material, and such agglutinins were produced in high potency by the writer with Hopkins. Subsequently in our own laboratory with Hopkins and McBurney, extensive experimentation on the production of antibodies with culture pallida was carried out.

We were able to show that not only were agglutinins formed by the immunization of rabbits with such cultures, but also that treponemacidal antibodies which were analogous to the ordinary bactericidal substances in such sera were present. Also, it was shown by cross agglutination and absorption experiments, that treponemata cultivated from various sources were related in group reactions.

Indeed, experiments done with cultivated treponemata (much facilitated by the discovery of a simple method of obtaining mass cultures of old strains) seemed at first very encouraging in that animals immunized with the cultures responded by powerful anti-

[112] Schereschewsky, J. *Deutsch. med. Woch.,* 1911, Vol. 37, Nos. 20 and 39.

[113] Mühlens. *Treponema Pallidum in v. Prowazek Handbuch der Pathog. Protozoen, i,* Barth, Leipzig, 1912; *Klin. Jahrb.,* Vol. 23, 1910, p. 339.

[114] Hoffmann. *Berl. klin. Woch.,* 1905, No. 46.

[115] Kolmer, J. A., Williams, W. W., and Laughbaugh, E. E. *Jour. Med. Res.,* Vol. 28, 1913, p. 345.

body formation. It was perfectly justified hope, therefore, that antibodies produced with these "attenuated" or rather "avirulent" strains might have some action on the virulent treponemata in luetic lesions. However, subsequent work in this direction disappointed such expectations. We may briefly review this work as follows:

The serum of rabbits immunized with "culture" pallida although potent against "culture" pallida, had no effect either in agglutinating the virulent organism from rabbit lesions, nor did it exert any protective influence when the virulent organisms were subjected to its action before injection.

Conversely, the serum of syphilitic rabbits showed but a very slightly increased agglutinating power for the "culture" pallida. This increase of potency in a few experiments was definite but very slight, a few of the syphilitic animals agglutinating as highly as 1:25 and 1:50, whereas most of the normal rabbits agglutinated in 1:10 and some of them 1:25.

Although Kissmeyer has recently reported that diagnostic use might be made of the fact that sera of syphilitic individuals agglutinated the "culture" pallida, we have tried this with a considerable number of cases and found that although the sera of tertiary syphilitics will sometimes agglutinate a little more highly than will the sera of normal individuals, yet many patients suffering from non-syphilitic diseases agglutinated as highly and almost as regularly as did the syphilitics.

We have come to the conclusion, therefore, as far as our work has gone, that in the syphilitic human being there is as little agglutinin formation against the "culture" treponema as there seems to be against the virulent organisms. If the slightly greater agglutinating power found in some of the tertiary syphilitics can be considered at all, the reaction is so feeble that it is negligible from the points of view either of diagnostic value or protective importance.

Furthermore, vaccination either intravenously or locally into the testis with cultures has thus far failed to protect rabbits against subsequent inoculation with virulent material, and passive immunization with sera produced with "culture" pallida has been without effect.

From all this it appears that the "culture" treponema has immunologically no relation to the virulent organism. It has lost its virulence completely, as six and more successive inoculations into rabbit testes have sufficiently demonstrated to us.

The sera produced by many injections of dead and living culture organisms have no effect whatever on the virulent organisms in vitro, and vaccination with it does not protect against subsequent infection with living treponemata.

The luetin reaction is the only method by which the relationship between the two is demonstrable at all, and there, too, we have to

reckon with what is generally spoken of as the non-specific increased sensitiveness of the syphilitic skin.

Were it not for the production of lesions with cultures in their early test tube generations by Hoffmann [116] and by Noguchi in a few experiments, one would be almost in doubt as to the identity of the virulent with the culture organisms.

The reactions in syphilis between the invading micro-organism and the invaded subject thus differ in certain fundamental premises from those prevailing in diseases caused by most bacteria. The treponema pallidum is an organism which, unlike many bacteria, is rarely subjected to the necessity of adapting itself to extra-corporeal existence during the interval between its passage from one host to another. It practically always infects directly, being inoculated from one human being to the next and has in consequence developed a very delicate parasitism not unlike that seen in certain trypanosome diseases of rats and that which we ourselves have observed in the well-known spirochete infections of white mice. A considerable percentage of laboratory white mice have been found to harbor actively motile spirochetes, often in considerable numbers in the blood and peritoneal fluid without there being any objective signs of illness in the animals. It is an instance of what has been spoken of by Bail as "infection without disease," and approaches what biologists speak of as symbiosis, except that the host in this case does not benefit in any way by the invasion. Even in the case of the mice, a certain amount of gradual injury, perhaps only metabolic, by the slow removal of nutritive material, is taking place. In syphilis the mutual adaptation may perhaps be less complete, a sufficient accumulation of the invaders and especially a mechanical injury of tissue cells, of closing of tissue spaces together with a certain amount of toxic action, leading eventually to pathological changes.

The virulent treponemata apparently do not arouse true antibody formation in any marked degree. When they have been cultivated and have become accustomed to the test tube conditions they entirely lose their virulence, are easily attacked by the active constituents of animal serum, and are probably amenable to phagocytosis. When such cultures, living or dead, are injected into animals they act like other specific protein antigens and incite the formation of antibodies. However, these antibodies have no effect whatever upon the virulent organisms.

In consequence, it cannot astonish us that all efforts at passive immunization with the sera of syphilitic man and animals, or with those of animals systematically treated with dead virulent materials, have been unqualified failures.

However, this does not preclude the theoretical possibility of active immunization or vaccination with such materials since, in this

[116] Hoffmann, W. H. *Deutsch. med. Woch.*, Vol. 37, 1911, p. 1546.

case, the antigen distributed from the points of injection might act upon tissue cells throughout the body. However, with the exception of the unconfirmed reports of Spitzer, all attempts to vaccinate either with dead virulent material, or with living and dead culture material, have been disappointing. The few experiments of Metchnikoff with virus "attenuated" by passage through monkeys have indeed seemed to indicate some possibility of approaching the subject from this direction, but these isolated observations have been very logically criticized by Neisser and should not bear too much weight. The observations were made on two cases only, both of them well along in life, and the validity of the important conclusions drawn rests entirely on the always problematical fulcrum of complete exclusion of previous syphilitic infection in the two subjects. Moreover, attempts in this direction would be fraught with a considerable amount of danger and it is therefore questionable whether experimentation along these lines is sufficiently promising to be justified. There is certainly no attenuation for man by passage through rabbits as has been sufficiently proven by a number of accidental infections, an instance of which in a laboratory attendant has been reported by Graetz and Delbanco.[117]

We may state, therefore, as safely summarizing our knowledge of the conditions in syphilis, that the resistance which undoubtedly develops during the course of the disease is one which depends upon reaction to the living virus only, cannot so far be produced in animals by systemic treatment with dead treponemata, and does not express itself in the formation of significant amounts of circulating antibodies analogous to those observed in bacterial diseases. Moreover, it is a well-known fact that the treponemata can continue to do injury to many organs and tissues at a time when reinfection by the paths of skin and mucous membranes is no longer possible.

How, then, are we to explain this peculiar state of affairs? A clue to the problem we think is found in the 20 rabbits which Hopkins, McBurney and the writer [118] inoculated into the testes after apparent recovery from a previous lesion. Ordinarily in rabbits, as we have stated before, no generalized resistance is developed during the disease, and the opposite testis can be successfully inoculated before, during, and after the existence of a lesion on the other side. In these rabbits it was found that testes that had apparently recovered from a previous lesion, were not subsequently as easily infected as were normal testes. It has seemed to us from this as well as from a careful study of the observations of other investigators, that resistance in syphilis was probably a matter of localized reaction. Tissues which have sustained active invasion with the living virus

[117] Graetz and Delbanco. *Med. klin.*, 1914, pp. 375 and 420.

[118] Zinsser, H., Hopkins, J. G., and Gilbert, R. *Jour. Exp. Med.*, Vol. 21, 1915, p. 213.

react and gain thereby a certain degree of resistance which expresses itself in a failure to react to subsequent inoculation. This would explain why in syphilis of the human being reinoculation is unsuccessful and reinfection of the skin and mucosæ does not occur spontaneously at a period later than the early secondary stages when the virus has become systematically distributed. It would furthermore explain why in this disease organ after organ may be pathologically involved when skin infection is no longer possible. This point of view is entirely in harmony, though perhaps from a slightly different aspect, with the skin immunity suggested by Kraus and Volk, where the resistance is attributed to the tissue as a whole rather than to a local cell group. The cells which have once reacted to the living virus no longer respond, *i. e.,* can no longer be injured for an indefinite period after recovery. However, the factors which lend them this resistance, whatever they may be, are not distributed to the blood stream in a way analogous to that in which antibodies are mobilized in bacterial diseases, and the effect of the resistance of the local area is not distributed to remote parts of the body. It is of course likely that a certain amount of phagocytosis of treponemata by the now resistant fixed cells may account for the absence of local injury. This, however, we have not yet been able to prove sufficiently, and further studies on this point are necessary. As far as any positive evidence can be adduced at the present time, the newly entering treponemata may not be entirely destroyed. It may well be that the tissues do not react and are in the state which Neisser calls "Anergie." The treponemata that enter during this period may nevertheless remain uninjured and be as capable of subsequently causing lesions in other locations as are those already present in the patient. This phase is being studied by comparative histological observation on the fate of treponemata which have been injected into normal tissues and into tissues rendered resistant by previous infection.

In animals like monkeys and man where generalization is rapid and apparently complete, the resistance becomes a general one. In animals like the rabbit in which the lesion—or in other words pathological response—occurs in a few organs only, the resistance is limited to the particular organ or organs that have previously developed a lesion.

It must not be forgotten that such a resistance probably persists for a limited time only, and does not imply the sterilization of the body and the complete destruction of the micro-organisms. These may, and probably do, remain alive and potent in various parts of the body, capable of again setting up new lesions in parts hitherto uninvolved or again susceptible after a diminution of their local, acquired resistance. That the virulent treponemata may remain thus latent, alive and virulent has been sufficiently shown in animal experimen-

tation by successful inoculation with tertiary lesions and by the frequent late accidents, especially of the nervous system, in individuals apparently cured or for a long time without symptoms.

It would seem when we analyze the conditions in syphilis, that complete sterilizing immunity or, in other words, complete cure, occurs but rarely without specific medicinal aid, and that the untreated syphilitic (if such an unfortunate individual exists in a civilized country) might go on to apparent cure, in that a general syphilization of his body would bring about a general resistance, but would always harbor virulent treponemata which could cause recrudescences in parts in which resistance was diminished, and eventually kill by degenerative processes in the central nervous system where many injuries cannot be compensated for as is possible in other organs.

The resistance which develops is apparently a new attribute only of the cell groups which have undergone direct reaction with the treponemata. This resistance may consist merely in the complete failure of the tissue cells to react to the virus, a sort of "tissue indifference" or "Anergie." It may be, however, and probably is, accompanied by a certain amount of active defense in the form of local phagocytosis of the treponemata by the fixed tissue cells.

CHAPTER XXII

NON-SPECIFIC EFFECTS OF PROTEIN AND PROTEIN DERIVATIVES. SERUM ENZYMES. LEUCOCYTIC ENZYMES.

THE INFLUENCE OF INJECTIONS OF NON-SPECIFIC SUBSTANCES UPON INFECTIOUS DISEASES

The therapy of infectious diseases has been very logically dominated in the past by attempts to increase resistance, either passively, by the injection of specific anti-sera, or actively, by treatment with bacterial antigens or their derivatives. The idea of specificity, in other words, has dominated such attempts almost exclusively. In spite of this, however, there has gradually grown in the minds of bacteriologists an impression that not all the effects of the injection of bacterial protein were purely specific. Jobling, who has recently summarized most of this work, calls attention to the fact that Matthes [1] as early as 1895 showed that the effects of tuberculin injections could be obtained equally well with deuteroproteose. From that time on, many observations have been made which show that profound physiological effects can often be produced in human beings and animals suffering from infectious diseases, if they are treated not with specific antigen but with proteins and protein derivatives of many sources.

The work that the writer did with Hiss on the injection of leucocytic extracts is a case in point. Similar in significance, probably, are the results obtained by the injection of substances such as the filtrate of bacterial cultures, commercially sold as phylacogens, the intramuscular milk injection practiced by Schmidt [2] and the injection of various ferments, reported by a number of writers throughout the literature of the past ten or fifteen years. Into the same category fall the favorable reports supposed to have been obtained in syphilitics with tuberculin reported by Blach.[3] Perhaps the clearest example of this principle has been obtained in connection with attempts at vaccine therapy in typhoid fever. Attempts to cure typhoid fever by the injection of typhoid bacilli date back to Fraenkel, who treated it in this way as early as 1893. Since then,

[1] Matthes, M. *Deutsch. Arch. f. klin. Med.*, Vol. 45, 1895.
[2] Schmidt, R. *Med. klin.*, 1910, No. 43.
[3] Blach, M. *Wien. klin. Woch.*, 1915, No. 49.

extensive studies have been made on this subject by Petruschky, Netter, and others. Ichikawa in 1912 and Boinet in 1914 obtained astonishing results by the use of sensitized vaccine, the former being the first to introduce the intravenous method of injection. The results of intravenous injection into a patient suffering from typhoid fever consisted in rapid falling of temperature, often followed by a chill, with occasionally rapid general improvement of the patient.

Gay, too, has made similar such observations by the methods of Ichikawa, and most of these writers were inclined to believe that the treatment was effective by some sort of specific reaction. Doubt has been cast upon this point of view, however, by observations such as those of Kraus. Ichikawa alone had some doubt of this, as is indicated by the fact that he injected typhoid bacilli into some of his paratyphoid patients with like results. Kraus subsequently obtained similar effects by injecting colon bacilli into typhoid patients and used non-specific bacterial virus with good results on cases of pyocyaneus infection and upon a single streptococcus puerperal septicemia. Leudke injected typhoid patients with non-bacterial proteose and Jobling and Petersen obtained very severe reactions in typhoid patients by injecting them intravenously with quantities of 1 to 2 c. c. of 2 per cent. solution of proteoses.

There seems to be little doubt of the fact that the injection of bacterial proteins intravenously produces a profound therapeutic effect without being in any but a minor degree specific. Boinet is still an adherent of specific reaction, believing that his typhoid patient was benefited by a rapid mobilization of antibodies and Gay had the idea that a specific hyperleucocytosis was the basis of improvement. This last phenomenon has been discussed in another place. We, ourselves, are inclined to believe that the specific reactions have little to do with the improvement in such cases and that the results are due to the injection of foreign protein and perhaps proteoses; the specific nature of the sources of these have little significance.

The entire problem of non-specific resistance and what is spoken of as protein therapy has recently been made the subject of a monograph by William F. Petersen.[4] Among the non-specific agents that have been used for various purposes in medicine, in addition to bacterial substances, Petersen lists normal serum, various proteins, egg albumen, milk, gelatin; protein-split products, proteoses, globin, pepton; enzymes, and cell products, such as trypsin, leucocyte extracts, autolysates of tumor tissue, colloidal metals, gold, silver, magnesium, platinum, mercury, zinc; hypertonic and hypotonic salt solution, sugar solution, distilled water, etc.

Whatever our opinion of the wisdom or therapeutic possibilities

[4] Petersen. "Protein Therapy and Non-Specific Resistance," Macmillan Co., New York, 1922.

of the administration of non-specific therapy of this kind may be, the fact remains that many of the agents used exert a very profound physiological action upon the body. The symptoms following injections will vary, both in degree and kind, with the nature of the material used, and the quantities injected. For details, we must refer the reader to Petersen's monograph. In most cases, however, we may state that the symptoms include a chill which may set in as early as 15 minutes after the injection of such materials as bacterial extracts or proteose solutions, but may be delayed for several hours. The same has been noticed after intramuscular milk injections. The temperature curve varies tremendously, but will usually rise, reaching its maximum within 4 or 5 hours. The pulse rapidly increases and the leucocytes go up. After the subsidence of the chill, there is a progressive decline of the blood pressure, and at the same time profuse sweating may be noticed. Nausea and gastrointestinal symptoms occasionally supervene. There may be great nervous irritability, and sometimes enlargement of the spleen. Albuminuria is irregular. There may be an increased lymph flow, and, as Jobling and Petersen ascertained, there may be a considerable change in the concentration of the serum enzymes. According to Petersen, the serum protease in patients almost invariably decreases after the shock, but later increases for a period of from 3 to 4 days. The serum peptidase usually increases in the cases that respond with improvement. Lipolytic enzymes showed no change, and the diastatic activity was usually diminished.

Many theories have been brought forward to explain the mechanism of the reaction. Attempts to explain it all by a mobilization of antibodies and eventually bringing it back into specific channels of reasoning, have not been adequate. It is not impossible, of course, that the entire process is related to a preliminary intoxication on the basis of anaphylatoxin formation, such as that more thoroughly dealt with in another chapter. Petersen seems to lay a considerable amount of stress upon the detoxicating effects of increased enzyme activity. Erepetase, that is, the ferment which is able to digest partially hydrolyzed proteins and is active at neutral reactions, is partially a detoxicating agent, and he considers a mobilization of this enzyme as being of distinctly beneficial significance, and never a factor in the production of an intoxication. As a further favorable effect we may consider the increased leucocytosis. Petersen also suggests a change in the permeability of the cell membranes in the direction of increasing the resistance of the cells to the toxic effect. The entire problem is still clothed in considerable mystery, and entirely too complex to be dealt with in this book. Petersen, himself, has appraised all the evidence on probable mechanism and states that the non-specific agents may all of them produce reactions that are funda-

mentally alike, but that the determining effects in different disease processes may vary considerably.

Various forms of this non-specific treatment have been utilized in arthritis, in acute fevers, like typhoid, paratyphoid, etc.; in gonorrhea and its complications; in anthrax, bacillary dysentery, erysipelas, influenza, scarlet fever, septicemias; in asthma, various diseases of the nervous system, and in skin diseases.

Before leaving this subject, it seems to us important to express some opinion as to the possibility of the treatment and the judgment to be employed in its use. Again quoting Petersen, we may say that "protein therapy offers a possibility, perhaps the most powerful method at our command, for the alteration of the current of cellular activity either in the direction of acceleration of function, or depression of function." Proper dosage is, therefore, of the greatest importance since over-dosage with a toxic agent may lead to a depression of function and great injury. His idea is that in order to intelligently apply the method in infectious diseases it is necessary to thoroughly realize that non-specific therapy is merely a method of stimulation of the cellular and humoral forces of the body for short periods, and is, therefore, useless if overdone or applied when the body is in a state of intense depression.

In the choice of substances for use, he states that for intravenous injection, protein-split products are more satisfactory than vaccines; if relatively mild reactions are desired, the various serums are useful; and where moderate general reactions are desired, intramuscular injections of boiled market milk are to be considered.

Even with the greatest precautions as to dosage and choice of material, there are definite contra-indications of which we know at the present time, and there may be a great many contra-indications that have not yet been revealed. The most serious contra-indication is the intense physical depression of a patient already severely ill from any disease. Furthermore, the history of serum sickness, asthma, urticaria, or other signs of hypersensitiveness should be regarded as contra-indications, as well as severe nervous diseases. Alcoholism, Petersen states, is an absolute contra-indication, pregnancy as well. In severe cardiac conditions, the greatest care should be observed. Diabetes is considered a contra-indication. In infectious diseases, like typhoid fever, the older cases towards the latter part of the normal clinical course, and the very septic cases should be excluded from the treatment.

Finally, we would like to state that while including this problem of non-specific therapy in this book because of its obvious bearing upon general problems of resistance, we do not think that the treatment, at the present time, has reached the stage at which it can be considered as anything but experimental, and should not be attempted by physicians who have not very thoroughly familiarized themselves

with clinical records, and who do not approach it very distinctly with the precautionary accuracy of a worker conscious that he is carrying out an experimental measure.

Serum and Leucocytic Enzymes.—In our section upon the nature of the precipitins, especially in the discussion of Gengou's conception of "albuminolysins," we have called attention to the probable significance of protein antibodies as a mechanism for the disposal of such foreign substances. In the bodies of the higher animals in which a special alimentary system, with its many digestive ferments, is well developed, it is most probable that the normal condition of digestion is one in which the foreign substances utilized for nutrition are completely split into their simpler components before they gain entrance to the circulation. Nevertheless, abnormal conditions or accidents, such as gastro-enteric diseases, digestive disturbances, and bacterial infections, may lead to a condition, probably frequent enough in ordinary life, during which such foreign substances may get into the blood stream without previous cleavage. The problem is to determine where and how such substances, protein or otherwise, are broken up so that they may be either assimilated or eliminated. We have referred in another place to the fact that foreign proteins may occasionally pass through the kidneys and be eliminated unchanged. This has been shown actually to occur by Oppenheimer, Ascoli, and others, but probably represents a very unusual state of affairs produced by special experimental conditions. As a rule these substances are disposed of within the body by chemical cleavage or by assimilation. It is more than likely, therefore, as has been emphasized by such workers as Jacoby, Salkowski, Wells, and others, that every fluid and cell in the body contains enzymes which, by their action upon proteins, fats, and carbohydrates, play an important part in the metabolism of the body. It has been known for a long time that the organs of animals removed from the body will undergo self-digestion by a process spoken of as autolysis. The action of bacteria as causing such autolysis can be excluded by covering organ emulsions with toluol, chloroform, and some other substances which have the peculiar property of preventing the growth of bacteria without in any way interfering with the action of enzymes. Thus, any organ of the body so treated will show rapid changes consisting in the splitting of its own proteins, fats, and carbohydrates. That such processes also go on in the living body is probable, although, as we have stated in an earlier chapter, it is a well-known fact that living cells oppose a more or less mysterious resistance to enzymic digestion, just as they oppose a similar resistance to bacterial invasion. The phenomenon is more fully discussed by Wells in his "Chemical Pathology," to which the reader is referred. It is not improbable that this resistance to invasion and enzyme attacks, spoken of vaguely as "vital resistance," can be analyzed into more exact factors, one of which seems to be

the question of reaction, digestion depending to some extent upon the reduction of alkalinity by the formation of acid in the tissue cells.

However, the physiological importance of tissue enzymes is a subject which we cannot go into in this connection, since the relationship of these enzymes to normal body metabolism is a subject more fully dealt with in text-books of physiology and is too far removed from the subject of our present discussion to warrant extensive analysis. It must, of course, be self-evident that the existence of cellular ferments of this nature must play an important rôle wherever and whenever cell death occurs, and since such cell death is an accompaniment of many phases of bacterial infection it may well be that the enzymes which destroy dead cells, whether they be bacterial or those of the body itself, contribute to the general pathological picture characterizing such diseases. Moreover, the importance of the enzymes is particularly enhanced by the knowledge we have gained from the work of Vaughan and others, who have shown that in the course of proteolytic cleavage toxic substances are liberated. It is such proteolysis which is the probable basis of the formation of the "anaphylatoxin" of Friedberger, the "proteotoxin" about which we ourselves have written, and the "sereotoxin" of Jobling and Petersen; and similar processes are involved in the toxic substances, studied by Whipple, which form in the intestine as a consequence of ligature of the gut. It is conceivable that wherever and whenever a proteolytic ferment reacts in the body with its substrate, toxic cleavage products result, perhaps in the form of albumoses, peptones, etc., which are rapidly absorbed and cause symptoms of varying intensity. In the chapter on anaphylaxis we have seen that many writers have attributed the injury occurring in this phenomenon to the formation of such split products and there is much evidence in favor of such a view, although the rapidity and vehemence of anaphylactic shock indicate that probably there are purely physical elements also involved which we do not as yet comprehend.

Of the cells in the body with which enzyme study has been most assiduously followed, the most important are the leucocytes. We have already had much to say in the chapters on phagocytosis of the digestive functions of the white blood cells. However, we dealt with them there chiefly from the point of view of their phagocytic and antibacterial functions. It will be useful to consider at a little greater length the importance of these cells from the point of view of purely enzymotic activity.[5]

As early as 1885 Hammarsten called attention to the fact that leucocytes aid in dissolving fibrin. Leber [6] subsequently studied pus

[5] See Wiens. *Ergebnisse d. Allg. Pathol.* Lubarsch & Ostertag, Vol. 15, 1911.

[6] Leber. *Entstehung der Entzündung.* Leipzig, 1891.

and found that it possessed a powerful digestive action for gelatin, fibrin, and other protein substances. He correlated his studies particularly with the processes of inflammation, and his work, as well as that of later investigators, brought into the foreground the fact that in the resolution of abscesses, especially of the staphylococcus variety, the leucocytes might play an important rôle in liquefying the necrotic tissue cells and bringing about the breaking down of the center of the abscess. Any one who has carefully studied the histology of a staphylococcus abscess can easily see the halo of disintegrating tissue lying just inside the ring of aggregated polymorphonuclear leucocytes. That these cells carry on an important part in the liquefaction is certain, although as yet we are not sure whether or not the proteolytic bacterial enzymes participate. Friedrich Müller,[7] in 1902, also studied these processes and attributed clinical significance to the proteolytic activities of the white blood cells in the resolution of the pneumonic lung. The study of inflammatory exudates by Opie [8] confirmed much of the preceding work and revealed that the ferments of all white blood cells were not the same. He found a leucoprotease which was contained in the polynuclear leucocytes which was active chiefly in a slightly alkaline medium, whereas the large mononuclear cells of uncertain origin which appear toward the end of an inflammatory process contained a protease which was active in weak acid. This differentiation of a leucoprotease and a lymphoprotease was more or less confirmatory of assumptions made earlier by Metchnikoff. It seemed probable that the acid protease noticed by Opie is specific to the large mononuclear cells of such exudates and is not common to lymphocytes in general, for the consensus of opinion of other workers seems to be that the small lymphocytes contain no proteolytic enzyme whatever. The study of tuberculous exudates particularly has failed to reveal proteolytic properties when only the characteristic small lymphocytes were present, although some writers, Bergell [9] especially, have shown that these cells contain a fat-splitting enzyme. Such lipase could also be determined in the press-juice of lymphoid organs, such as the spleen and lymph nodes. It is not improbable that the characteristic dry abscess or "caseous" abscess accompanying typical tuberculous lesions owes its peculiar histological characteristics to the lack of proteolytic enzymes.

Jochmann [10] and his collaborators have extensively studied leucocytic extracts in this regard and, curiously enough, found that the proteolytic enzymes of leucocytes of which we have been speaking

[7] Fr. Müller. *Verh. d.* 20 *Kongress f. innere Mediz.*, Wiesbaden, 1902.
[8] Opie. *Jour. of Exp. Med.*, Vols. 7 and 8, 1905 and 1906.
[9] Bergell. *Münch. med. Woch.*, 1909.
[10] Jochmann. For summary of his work and literature see *Kolle u. Wassermann,* 2nd ed., Vol. II, 2.

can be found only in the cells of man and monkeys and, to a slight degree, in dogs. In these species only, according to Pappenheim,[11] do the leucocytes contain true neutrophile granules and it is therefore a possibility that the neutrophile granules and the enzyme action are related to each other. The leucocytes of rabbits do not apparently contain protease, but recently in our laboratory Mrs. Parker and Miss Francke have shown that rabbit leucocytes contain erepsin.

The proteolytic enzymes of leucocytes curiously enough continue their activity at temperatures as high as 55° C., a fact which makes it possible to investigate their activity at temperatures at which most bacteria will no longer grow and functionate, and this incidentally facilitates sterile experimentation. Their activity is astonishing in that Jochmann found instances where dilution even as high as 500-fold with salt solution did not completely eliminate the proteolytic activity of pus. The simplest method of demonstrating such action is to place pus or washed leucocytes, in droplets, upon the surface of plates of Loeffler's coagulated blood serum, such as that used in the cultivation of diphtheria bacilli. On such plates, small indentations rapidly give evidence of the liquefaction of the coagulated protein.

Casein also can be used as an indicator of proteolytic digestion, a casein solution being made by dissolving a gram of casein in 100 c. c. of N/10 NaOH solution and neutralizing this to litmus with deci-normal HCl. A very curious property of the leucocytic ferment has been described by Jochmann and Ziegler, who reported that preservation in 10 per cent. formalin solution will long preserve the fermentative activities of the cells.

It was formerly supposed that the proteolytic properties of leucocytes were more or less pathological. But we have since learned that these powers are common to leucocytes both in health and in disease. The older idea was due to the fact that the earliest investigations of the ferments were made in connection with myelogenous leukemia. Jochmann and Ziegler studied the organs of patients dead of this disease and found that the bone-marrow, spleen, and lymph nodes of such cases had powerful proteolytic properties in contrast to the very weak activities in this regard of normal organs. The proteolytic activity was more or less in direct proportion to the degree of myelogenous infiltration. In lymphatic leukemia no such activity was discovered. In consequence there has been a tendency to draw a fundamental physiological distinction between these types of cells, a distinction which would have considerable importance in discussions of their origin and significance.

Jochmann has an idea that the activities of these ferments in connection with tissue destruction and other pathological conditions where they become active, may be an important phase in the production of fever.

[11] Pappenheim. Cited from Wiens, *loc. cit.*

An important question which immediately arises is whether these ferments can be identified with the bactericidal substances described in a preceding chapter as existing within leucocytes. According to Jochmann the enzyme extracts have no bactericidal properties. Indeed, it is a curious fact that living bacteria oppose a very powerful resistance to digestion by these and other ferments. Kantorowicz [12] who has studied this particularly, has shown that living bacteria contain a very powerful antiferment which is similar to the antiferment presently to be described for blood serum. He has shown that living bacteria cannot be digested by trypsin, a resistance which is lost when the bacteria are heated to 80° C., and an extract of living bacteria will prevent the tryptic digestion of the heated bacteria. It will appear, therefore, that in the process of phagocytosis the bacteria are first killed by the bactericidal substances contained in the cells and are later digested by the leucoprotease.

That the lymphocytes contain lipase has been mentioned above and since these cells are specifically accumulated about tuberculous foci it has been many times suggested that their function is particularly directed against these acid-fast bacteria in whose constitution the presence of waxes and fats plays such an important rôle. It is a fascinating thought, though entirely conjectural, that perhaps the specific benefit of feeding fats in tuberculosis may have some basis in the possible increase of lipolytic ferments which appear in response to the stimulation of introducing larger quantities of fats.

It is hardly necessary to reiterate here the possible importance of these cellular enzymes in the many different phases of the absorption of larval organs which occurs in the lower animals in the course of normal development, or of dead tissue in mammalia in disease and in the processes of senescence.

In addition to proteolytic enzymes, it has been long known that the leucocytes also contain an oxidase. This enzyme can be demonstrated by the well-known guaiac test, in which tincture of guaiac is added to leucocytic exudates or pus, and a blue color results as a consequence of oxidation of the guaiac. The oxidizing ferment is apparently limited to the polynuclear leucocytes. Exactly what its significance is we do not know but it is highly probable that it plays an important part in the metabolism of the leucocyte.

It has only recently become clearly apparent that the function of intracellular digestion of foreign substances, such as invading bacteria, is not limited to the circulating leucocytes but is to a very large extent also carried on by the fixed cells of organs. Studies by Wyssakowitz,[13] by Kyes,[14] by Bartlett,[15] and, recently, by Hopkins

[12] Kantorowicz. *Münch. med. Woch.*, Vol. 56, 1909, p. 897.
[13] Wyssakowitz. *Zeitschr. f. Hyg.*, Vol. 1, p. 1.
[14] Kyes. *Jour. of Inf. Dis.*, Vol. 18, 1916, p. 277.
[15] Bartlett. *Jour. Med. Res.*, Nov. 5, 1916, p. 465.

and Parker,[16] have shown that in the course of many bacterial infections the greater proportion of invading bacteria is taken care of by cells of the liver, spleen, and especially the lung. Streptococci injected into rabbits rapidly disappear from the circulation, largely because they are taken up very actively by the (probably) endothelial cells of the lung where apparently they are killed, their digestion taking place within the cells very slowly. The endocellular enzymes bringing this about can, of course, not be separately studied as can those of leucocytes, for obvious technical reasons.

While the cellular enzymes have been very carefully studied, it is only of relatively recent years that much attention has been paid to the enzymes present in the circulating blood. This is to some extent due to the fact that German writers especially have taken for granted that enzymes in the blood were nothing more or less than liberated leucocytic enzymes, and also because the activity of the circulating enzymes has been largely masked by the existence of a powerful antiferment which must needs be there for physiological reasons to prevent injurious autodigestion. We have already mentioned the fact that the study of pathological conditions, such as myelogenous leukemia, was the point of departure for work upon the leucocytic ferments, and this, to a certain extent, was also the beginning of the studies on serum ferments. When blood serum is incubated with various substrates, it is not unlikely, as we shall see in our subsequent discussion of the Abderhalden reaction, that the antiferment is absorbed and thereby the proteolytic and other ferments of the blood are liberated. There is normally an excess of antiferment in the blood, and normal human serum usually does not contain strong proteolytic enzyme. These two factors together therefore prevent powerful proteolysis by normal serum. There are conditions, however, under which the enzyme contents of the blood are increased. Thus, the sera of pregnant women have been shown to be relatively rich in proteolytic properties, and such an increase is also present during starvation, as Schultz,[17] and Heilner and Poensgen [18] have shown. The same thing has been noted in cases of pneumonia, in which condition Falls [19] has shown a sudden diminution of this enzyme at the time of crisis, and the same author has made analogous observations in the fluctuation of serum protease in malaria, typhoid fever, and some other diseases.

The lipolytic activity of the serum has been found increased in syphilis, in diseases involving liver function, such as chloroform poisoning, and in a number of other conditions. In syphilis, indeed,

[16] Hopkins and Parker. In press at present writing.
[17] Schultz. *Deutsch. med. Woch.*, No. 30, 1908; *Münch. med. Woch.*, Vol. 60, 1913.
[18] Heilner and Poensgen. *Münch. med. Woch.*, Vol. 61, 1914.
[19] Falls. *Jour. of Inf. Dis.*, Vol. 16, p. 466.

the increased lipase contents have been held responsible for the Wassermann reaction by a number of writers. They believed that the lipolytic ferment acting upon the lipoid antigen produced fatty acid which by increasing the H-ion contents rendered the complement inactive. This to a certain extent was strengthened by the knowledge that in the Wassermann reaction the end-piece of complement remained free. However, we ourselves in hitherto unpublished experiments have been able to convince ourselves that there is no relationship between lipase contents and Wassermann positiveness.

Activity of intravascular enzymes of any kind may perhaps be going on constantly in the normal course of life as a part of general body metabolism; yet it is probable that any great extent, especially of proteolytic action, would be incompatible with health or even life. The work of Vaughan, of Friedberger, and others has shown us that in the course of proteolysis toxic cleavage products, probably of the albumose variety, are liberated. This we have seen has been made the basis of some theories of anaphylaxis, and the "split products" of Vaughan, the "anaphylatoxin" of Friedberger, the "proteotoxins," as we ourselves have chosen to call them, and the "serotoxins" of Jobling and Petersen, all are probably the results of such proteolysis. Indeed, as we have seen, the incubation of almost any tissue substrate with fresh serum will result in this proteolytic change and even in the formation of toxic substances. It was at first thought that the toxic split products were derived from the substrate, but we have seen in another chapter that the mechanism is probably one in which antiferments are removed by the substrate, and the serum-protease is liberated to act upon the serum protein itself. This was undoubtedly the case in such instances as those in which anaphylatoxin was obtained by digestion of serum with kaolin, barium sulphate, tubercle bacilli, etc. Now it appears that the injection of almost any substance, and especially of bacterial proteins, produces a sharp rise of enzymes in the blood. This seems to be true not only of proteolytic enzymes but of lipolytic and amylolytic ferments as well. Ferments so stimulated are entirely non-specific. This greater serum activity after protein injection may be due both to a direct increase of production and a simultaneous diminution of antiferments, by means of which activity hitherto inhibited is allowed to run riot. The result may be in some respects beneficial and in other respects injurious. The marked increase of the enzymes may aid in rapidly disposing of the unchanged foreign substance, and perhaps the beneficial effects following the injection of typhoid vaccines into patients suffering from this disease may be due to the mobilization of ferments as suggested by Jobling and Petersen.[20]

On the other hand, the proteolytic activity may result in protein cleavage, by means of which albumoses, etc., are liberated in greater

[20] Jobling and Petersen. *Jour. A. M. A.*, Vol. 65, 1915, p. 515.

or smaller quantities which act injuriously and lead to clinical symptoms of various kinds. That fever may perhaps be explained by poisoning with albumoses so produced has already been suggested by Jochmann, and that albumoses are present in various products of suppurative inflammation has also been shown. It has been shown by Pfeiffer and Jarisch [21] and others, that fluctuations in proteolytic enzymes accompany anaphylaxis. Jobling and Petersen [22] have formulated a theory of anaphylaxis based upon studies of serum protease. They observed that during the course of sensitization there occurred a gradual mobilization of non-specific protease which increased in intensity up to the time of maximum sensitization. They attributed acute shock to the fact that on the second injection there occurs an instantaneous mobilization of large amounts of non-specific protease together with a decrease in antiferment and an increase in non-coagulable nitrogen and amino-acids, and they believe that the cause of the acute intoxication is the rapid cleavage of the serum protease. The specific element they believe consists in the rapid mobilization of the ferments and the colloidal serum changes which bring about the change in antiferment titre. Fascinating as this theory is, and although it has a number of things in common with our own ideas concerning the colloidal balance in the plasma which ordinarily prevents the rapid union of antigen with antibody, we feel that recent knowledge concerning the essentially cellular nature of anaphylaxis in the guinea pig prevents its adoption. Furthermore, we believe that the specific element of anaphylaxis is insufficiently explained by Jobling and Petersen's conclusions.

Again the question arises: Are these serum proteases in any way to be identified with bacteriolytic antibodies or with alexin or complement? It is more than likely that no such relationship exists. In the first place, the seroproteases are non-specific, and it has been shown that in the course of bacteriolysis no increase in non-coagulable nitrogen occurs. Moreover, Jobling and Petersen,[23] to whom we owe much of our recent knowledge on this subject, have shown that the treatment of bacteria with complement alone or with complement together with human serum renders them more resistant to proteolysis, probably owing to the absorption of antiferments from the blood serum. The identification of complement with lipase has been suggested. The idea gains likelihood from the fact that both complement and lipase are destroyed by lipoid solvents and that many of the substances upon which complement acts, such as red blood cells, contain lipoid constituents. On the other hand, no definite knowledge is available in this regard, and it has been so far impossible to prove even the enzymotic nature of complement, though this, at least, seems

[21] Pfeiffer and Jarisch. *Zeitschr. f. Imm.*, Vol. 16, 1912.
[22] Jobling and Petersen. *Jour. Exp. Med.*, Vol. 20, 1914.
[23] Jobling and Petersen. *Jour. Exp. Med.*, Vol. 20, 1914, p. 321.

likely. The peculiar resistance exhibited by bacteria to digestion by ferments has already been alluded to.

It develops therefore that the antiferments in the blood are extremely important factors in maintaining the balance of enzyme activity, and in consequence the antienzymatic properties of serum have been investigated by many workers. For, depending upon their fluctuation, cleavage processes are permitted or prevented from taking place in the circulating blood. The earliest workers concerned themselves largely with the phenomenon that blood serum would prevent the proteolytic activity of leucocytes. They utilized the observation therapeutically by injecting serum into suppurating abscesses for the purpose of preventing tissue destruction by leucoprotease and increasing the ability of the body to limit the processes. Indeed it may be that this form of therapy in chosen cases may still have possibilities.

Later investigators studied antiferment fluctuations in disease. In septic conditions, Wiens [24] claims to have noticed a diminution of serum antiferments. Similar observations have been made after shock in anaphylaxis by Pfeiffer and Jarisch. In patients with cancer, Landois [25] claimed to have noticed a marked increase in antiferments, which for a time was believed to be sufficiently regular to have diagnostic significance. Brenner [26] noticed the same phenomenon in severe anemias. In cachexia the same phenomenon has been noticed and fluctuations in this constituent have been noticed in a great many different diseases, such as diabetes, pneumonia, etc., without our being at present in possession of any definite or correlated knowledge of the principles upon which these fluctuations depend.

Earlier observers believed that the anti-ferment in the blood was a function of the albumin fraction of the serum. More recent workers have attributed it to lipoid constituents. Schwarz,[27] Sugimoto,[28] and Kirchheim suggested the lipoid idea because of the fact that the antiferment properties were destroyed by lipoid solvents. They did not settle the question satisfactorily because their results were inconsistent, but Jobling and Petersen [29] found that antiproteolytic activity of blood serum was almost wholly inhibited when sera were extracted for several days with chloroform or ether at room temperature or with chloroform in the incubator for an hour. The antiferment could be recovered from the ether and chloroform solution, and its action could be destroyed by incubation with iodin or

[24] Wiens. *Münch. med. Woch.*, No. 53, 1907.
[25] Landois. *Berl. klin. Woch.*, No. 10, 1909.
[26] Brenner. *Deutsche med. Woch.*, No. 9, 1909, and *Mediz. Klinik*, No. 28, 1909.
[27] Schwarz. *Wien. klin. Woch.*, 22, 1909.
[28] Sugimoto. *Arch. f. Exp. Path. u. Pharmakol.*, Vol. 74, 1913.
[29] Jobling and Petersen. *Jour. Exp. Med.*, Vol. 19, 1914, p. 480.

potassium iodid. They believed this to be due to the saturation of the free carbon atoms. Furthermore, the same authors showed that toxic substances analogous to anaphylatoxins could be produced in serum when incubated with chloroform. The lipoidal nature of the antiferments is a likely one although it does not account for the fact noticed uniformly by all workers that the antitryptic activity can be entirely eliminated by heating the serum to 70° C. for a half hour. This was the observation which at first gave rise to the idea that the antiferment was in itself an enzyme. For this phenomenon of heat liability no satisfactory explanation has as yet been advanced.

The Abderhalden Reaction.—The recent researches of Abderhalden [30] upon the intravascular digestion of foreign substances introduced into animal bodies promised to have considerable bearing upon problems of immunity. Abderhalden, whose work we cite chiefly from his monograph, "Die Schützfermente des tierischen Organismus," took as his point of departure the conception that the animal body must necessarily preside over a mechanism whereby it can assimilate foreign substances which obtain entrance unchanged into the circulation. Abderhalden believes that this process depends upon the mobilization of "protective ferments," a term which he borrows from Heilner,[31] and suggests the possibility that these ferments may originate in the leucocytes.

Experimentally Abderhalden approaches his problem by determining the presence of specific ferments in the blood of animals into which various foreign substances have been introduced by paths other than the alimentary canal. For this purpose he has developed a number of methods, the most important of which are his optical method and his dialysis method. The optical method used for the determination of the proteolytic properties of the serum depends upon the fact that many of the amino-acids are optically active. Moreover, most of these substances are chemically known and their optical activity determined, so that it is possible to take blood serum which is to be examined for its contents of particular ferments, mix them with a suitable protein, or preferably a polypeptid, and determine with a polariscope the rotation which takes place. We will not go into the technique of this method more extensively because we have no personal experience with it, and the method is one of such delicacy that it is best obtained directly from Abderhalden's original publications.[32] His dialysis methods depend upon placing the blood serum and fermentable substance into dialyzing bags, sus-

[30] Abderhalden. "Schützfermente des tierischen Organismus," Springer, Berlin, 1912.

[31] Heilner. Cited from *Abderhalden Zeitschr. f. Biol.*, Vol. 50, 1907.

[32] See especially Abderhalden, Hoppe-Seyler, *Zeitschr. f. physiol. Chemie*, Vols. 60, 65, and 66; also "Handbuch der Biochem. Arbeitsmethoden," Vol. 5, 1911, p. 575.

pending these into distilled water, and determining the presence of peptone, amino-acids, or total nitrogen in the liquid outside of the bag after definite intervals of time.

By these and other methods Abderhalden [33] has carried out tests with a large number of different substances. Experimenting first with proteins, he injected egg albumen, horse serum, silk peptone, gelatin, edestin, casein, etc., into dogs and rabbits, then, several days later, bled the animals and mixed 0.5 c. c. of the serum with 0.5 c. c. of a solution of the respective substances which had been injected. He found in such cases that definite proteolytic action was exerted upon the injected substances by the active serum of a treated animal, whereas, in the case of most of the substances used, the normal serum possessed no proteolytic action whatever. These results were consistently obtained both by the dialysis and by the optical methods. It should be especially noted that the ferments studied by Abderhalden were not as specific as are the antibodies which we have discussed in another place. For Abderhalden found that the serum of an animal treated with proteins developed enzymes which were active, not only against the particular protein used for injection, but rather against proteins in general. They were specific only in that, when produced with proteins, they were not active against fats or carbohydrates. This is especially important in connection with the recent discussion concerning the identity of Abderhalden's protective ferments and the specific protein antibodies.

In later experiments Abderhalden claimed to show further that similar ferments could be induced in animals by treatment with carbohydrates and with fats. The serum of normal dogs is not capable of splitting cane sugar. However, the blood serum or plasma of a dog that has been treated with cane sugar develops the property of inverting the cane sugar into dextrose and fructose within fifteen minutes after injection. This could easily be determined both by putting together the serum with cane sugar and determining the increase of reducing powers, and by means of subjecting such active plasma or serum, together with saccharose, to polariscope examination.

The earlier experiments with fats were negative because the simple method of titration for fatty acids proved insufficient as an indicator of activity. However, Abderhalden succeeded in determining fat splitting properties in the blood of treated dogs by using the method of Michaelis and Rona.[34] The presence of fats largely increases the surface tension of mixtures, and their cleavage in such mixtures consequently leads to reduction of this tension. Utilizing this principle, Abderhalden claims to have determined that the paren-

[33] Abderhalden. "Schützfermente," p. 49.
[34] Michaelis and Rona. Cited from Abderhalden, *loc. cit.*

teral introduction of fats into dogs is followed by a reactionary increase of lipases.

The general conclusion drawn from Abderhalden's researches is this: When any foreign substances, protein, carbohydrate, or fats, gain entrance to the circulation of an animal, the animal body reacts by the mobilization of ferments or enzymes specifically capable of reducing these substances to assimilable form. It is likely that these ferments represent a mobilization of substances normally present but not concentrated in the blood stream under ordinary conditions, since they appear with a speed out of all proportion to that obtaining in the case of the antibodies discussed in another place. In one case cited by him a dog injected on November 25th, 29th, and December 4th showed powerful peptolytic serum properties on December 6th. Apparently the injection of homologous proteins into animals (i. e., rabbit serum into rabbits, etc.) does not incite reaction.

These enzymes seemed to differ from specific antibodies in that they did not react solely with the substance injected, but also with other substances belonging to the same chemical group. Other differences from antibodies are the rapid appearance of the ferments after treatment and their rapid disappearance after the inciting stimulus is removed. Thus Abderhalden reports that the enzymes found in a case of pregnancy disappeared within eight days after abortion or child birth.

It is plain that these researches of Abderhalden offer many opportunities for diagnostic utilization, and he has applied them to the diagnosis of pregnancy. In this condition substances from the chorionic villi get into the blood. These, according to Abderhalden, may be looked upon as in a certain sense foreign in nature, and must be chemically disintegrated by the body. In consequence it is likely that the ferments which accomplish this would appear in the sera of pregnant individuals and could be determined by his methods. When he prepared peptone from the placental substances of human beings and allowed the blood plasma of normal individuals to act upon it, observing it both by the dialysis and the optical method, no peptolytic action could be observed. However, when the plasma of pregnant women was used proteolytic action was determined. In these cases the ferment seemed to be specific for peptones produced from placental tissue both in animals and human beings, but did not act upon casein, gelatin, or other proteins. There are certain technical difficulties connected with the production of a test material from the placental tissue which render this method difficult. For their more detailed description we refer the reader to the original articles.

The Abderhalden reaction and its various logical ramifications promised for some time to bring an entirely new and important factor into the field of immunology, namely, that specific ferment mobilization in definite response to the introduction of various substances,

and yet distinct from the specific antibody heretofore known. The reaction rapidly became the subject of active research mostly of a clinical nature, and carried out with insufficient critical judgment. This literature is extensive and, because almost entirely refuted at the present day, is hardly worth citing. In this country the reaction has been carefully studied by a great many workers, more especially by Bronfenbrenner, and Jobling, Petersen and Eggstein. From all these studies, we may say for the sake of brevity, it has become clear that the enzymes involved in the Abderhalden reaction are probably not specific as supposed by this writer, that during the reaction the tissues employed (that is placenta, etc.), act by absorbing antienzymes from the serum. In consequence the ferments in the serum act upon the serum protein itself which therefore becomes the substrate of the cleavage product determined as a result of the reaction. This robs the reaction of any claim to the specific and diagnostic value attributed to it by Abderhalden and his collaborators, and as a final result of these extensive and intricate researches we have merely a better understanding of the non-specific enzyme activity of normal serum.

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